Departamento de Farmacología y Terapéutica
Facultad de Medicina
Universidad Autónoma de Madrid

Regulation of vascular COX-2, NOX-1 and NOX-4 expression by angiotensin II and interleukin 1β. Role of HuR and implication in cell migration

PhD THESIS

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Departamento de Farmacología y Terapéutica
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CERTIFICAN, que Doña Andrea Aguado Martínez ha realizado bajo su dirección el presente trabajo: *"**Regulation of vascular COX-2, NOX-1 and NOX-4 expression by angiotensin II and interleukin 1β. Role of HuR and implication in cell migration**",* como Tesis para alcanzar el grado de Doctor por la Universidad Autónoma de Madrid.

Para que conste a efectos oportunos, expiden y firman la presente en Madrid a 7 de Noviembre de 2014.

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Investigar es ver lo que todo el mundo ya ha visto y pensar lo que nadie ha pensado todavía

Albert Szent-Györgyi (1893-1986)

La felicidad humana generalmente no se logra con grandes golpes de suerte, que pueden ocurrir pocas veces, sino con pequeñas cosas que ocurren todos los días.

Benjamin Franklin (1706-1790)

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Abbreviations

AA: arachidonic acid

AC: Adenylyl cyclase

ACE: Angiotensin converting enzyme

ACEI: ACE inhibitors

AngI: Angiotensin I

AngII: Angiotensin II

AP-1: Activator protein-1

ApoE: Apolipoprotein E

ARB: AngII receptor blockers

AREs: Adenylate- and uridylate-rich elements

AT_1R: Angiotensin II type 1 receptor

AT_2R: Angiotensin II type 2 receptor

BH4: Tetrahydrobiopterin

Ca^{2+}: Calcium

CaM: Calmodulin

cAMP: Cyclic adenine 3', 5'- monophosphate

Cdk: Cyclin-dependent kinase

Chk2: Checkpoint kinase 2

COX-1: Cyclooxygenase 1

COX-2: Cyclooxygenase 2

CREB: cyclic AMP-responsive binding protein

cPGES: Cytosolic prostaglandin E_2 synthase

DAG: Diacylglycerol

DP: PGD_2 receptor

ECM: Extracellular matrix

EGFR: Epidermal growth factor receptor

Egr-1: Early growth response protein 1

ELAV1: Embryonic Lethal, Abnormal Vision, Drosophila-like 1

Elf-3: E74-like factor 3

Elk-1: ETS domain-containing protein 1

eNOS: Endothelial nitric oxide synthase

EP: PGE_2 receptor

ER: Endoplasmic reticulum

ERK1/2: Extracellular signal-regulated kinase 1/2

ETS-1: Erythroblast transformation-specific 1

FP: $PGF_{2\alpha}$ receptor

GPCRs: G protein-coupled receptors

GPx: Glutathione peroxidase

GR: Glutathione reductase

GRK: G protein-coupled receptor kinase

GSH: Glutathione (reduced)

GSSG: Glutathione (oxidized)

H_2O_2: Hydrogen peroxide

HIF: Hypoxia-inducible factor

HuR: Human antigen R

IL: Interleukin

IL-1β: Interleukin-1β

IL-1R: IL-1 receptor

IL-1Ra: IL-1R antagonist

IL-1RAcP: IL-1 receptor accessory protein

IP: PGI_2 receptor

IP_3: Inositol trisphosphate

IRAK: IL-1R-associated kinase

JAK: Janus kinase

JNK: c-Jun N-terminal kinase

MAPK: Mitogen activated protein kinase

MLC: Myosin light chain

MLCK: Myosin light chain kinase

mPGES: Microsomal prostaglandin E_2 synthase

MyD88: Myeloid differentiation factor 88

NADPH: Nicotinamide adenine dinucleotide phosphate (reduced)

NF-κB: Nuclear factor κ B

NO: Nitric oxide

NOS: Nitric oxide synthase

$O_2^{\bullet -}$: Superoxide anion

$OH^{\bullet}$: Hydroxyl radicals

$ONOO^-$: Peroxynitrite

p38 MAPK: p38 Mitogen activated protein kinase

pCaMKII: Ca^{2+}/CaM-dependent protein kinase II

PDGF: Platelet-derived growth factor

PG: Prostaglandin

PGE_2: Prostaglandin E_2

PGES: PGE_2 synthase

$PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$

PGG_2: Prostaglandin G_2

PGH_2: Prostaglandin H_2

PGI_2: Prostacyclin

PGIS: Prostacyclin synthase

PI3K: Phosphoinositol-3 kinase

PIP_2: Phosphatidylinositol 4,5-bisphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLA_2: Phospholipase A_2

PLC: Phospholipase C

PPAR-γ: peroxisome proliferator-activated receptors-γ

RAAS: Renin-angiotensin-aldosterone system

RIP: Receptor interacting protein

ROS: Reactive oxygen species

STAT: Signal transducers and activators of transcription

SHR: Spontaneously hypertensive rats

SOD: Superoxide dismutase

TAK: TGF-β-activated protein kinase

TRAF6: Tumor necrosis factor receptor-associated factor 6

TGF-β: Transforming growth factor-β

TNF-α: Tumor necrosis factor-α

TP: TXA_2 receptor

TXA_2: Thromboxane A_2

UTR: Untranslated region

VEGF: Vascular endothelial growth factor

VSMC: Vascular smooth muscle cells

WKY: Wistar Kyoto rat

XO: Xanthine oxidase

Abstract

Prostanoids and reactive oxygen species (ROS) are implicated in the vascular structural and functional alterations associated with cardiovascular diseases. Angiotensin II (AngII) induces vascular inflammation through increased production of cytokines such as interleukin 1β (IL-1β). Both AngII and IL-1β stimulate the expression of pro-inflammatory enzymes including cyclooxygenase 2 (COX-2) and NADPH oxidases increasing the production of prostanoids and ROS. Among the NADPH oxidase isoforms, NOX-1 and NOX-4 appear to be particularly important in vascular smooth muscle cells (VSMC). Human antigen R (HuR), a RNA binding protein, is able to stabilize different mRNAs favoring the pro-inflammatory status in pathological conditions. However, to our knowledge, no studies have evaluated whether post-transcriptional mechanisms mediated by HuR participate in the control of COX-2 or NOX expression in VSMC.

The aim of this PhD Thesis was to investigate transcriptional and post-transcriptional mechanisms implicated in the regulation of COX-2, NOX-1 and NOX-4 expression in VSMC stimulated with AngII and IL-1β. In addition, the role of this regulation in vascular remodeling was evaluated. In line with this general aim, the PhD Thesis was divided into two specific aims that provided the following results and conclusions:

1. HuR is involved in the stabilization of COX-2 mRNA in cancer cells and AngII is able to increase COX-2 mRNA stability in vascular cells. In the first part of the PhD Thesis, we investigated the contribution of HuR upon COX-2 expression induced by AngII and IL-1β and its consequences on VSMC migration and vascular remodeling. The main findings were: 1) In VSMC, AngII potentiated COX-2 and tenascin-C (TN-C) expressions and cell migration induced by IL-1β. 2) The effect of AngII on IL-1β-induced COX-2 expression was accompanied by increased COX-2 3'UTR reporter activity and mRNA stability occurring through cytoplasmic HuR translocation and COX-2 mRNA binding. These effects were blocked by ERK1/2 and HuR inhibitors. 3) VSMC migration induced by AngII+IL-1β was reduced by blockade of COX-2, TXAS, ERK1/2, HuR, TP and EP receptors and was stimulated by PGE_2 and TXA_2. 4) COX-2, mPGES-1,

TXAS and HuR expressions were increased in the aorta from AngII-infused mice and in carotid-ligated arteries. 5) TN-C expression and vascular remodeling induced by AngII infusion were abolished by a COX-2 inhibitor and by mPGES-1 deletion. These results demonstrate that the synergistic induction of COX-2 by AngII and IL-1β in VSMC involves HuR through an ERK1/2-dependent mechanism. Moreover, we demonstrate that the HuR/COX-2 axis participates in cell migration and vascular damage.

2. HuR-dependent post-transcriptional mechanisms are implicated in gene expression in inflammation; however, its involvement regulating NOX expression has not been studied yet. In the second part of the PhD Thesis, we explored transcriptional and post-transcriptional mechanisms involved in the effects of AngII and IL-1β on NOX-1 and NOX-4 expressions in VSMC and their implications in cell migration. The main findings were: 1) IL-1β increased NOX-1 expression, NADPH oxidase activity and ROS production, effects potentiated by AngII. Conversely, IL-1β decreased NOX-4 expression and H_2O_2 production that were unaffected by AngII. 2) AngII+IL-1β interfered with the decay of NOX-1 mRNA and promoted HuR binding to NOX-1 mRNA. Moreover, HuR blockade reduced AngII+IL-1β-induced NOX-1 mRNA stability and levels. 3) NOX-1 and HuR blockade reduced the AngII+IL-1β-induced NADPH oxidase activity and ROS production. 4) The decreased NOX-4 expression induced by IL-1β was blocked by a protein synthesis inhibitor and was accompanied by a decrease in 200 bp proximal promoter activity. 5) AngII and/or IL-1β increased VSMC migration that were prevented by NOX-1 and HuR blockade and were augmented by NOX-4 overexpression. Thus, HuR-mediated NOX-1 mRNA stabilization is partially responsible for AngII+IL-1β-dependent NOX-1 expression and ROS production in VSMC whereas transcriptional mechanisms are involved in decreased NOX-4 expression induced by IL-1β. Regulation of NOX-1/4 contributes to VSMC migration, important process in inflammatory conditions.

In summary, in this PhD Thesis we present evidences that the combination of AngII and IL-1β, two inflammatory stimuli important for cardiovascular diseases, synergistically induce the expression of two major pro-inflammatory proteins COX-2 and NOX-1 that in turn participate in VSMC migration and vascular remodeling. We also demonstrate for the first time that the RNA binding protein HuR is key to increase the mRNA stability of both genes, thus participating in cell migration induced by inflammatory stimuli. We believe that HuR/COX-2/NOX-1 pathways might be novel pharmacological targets to prevent or regress vascular remodeling associated to cardiovascular diseases.

Resumen

Los prostanoides y las especies reactivas de oxígeno están implicadas en las alteraciones vasculares estructurales y funcionales asociadas a enfermedades cardiovasculares. Angiotensina II (AngII) induce inflamación vascular a través de un aumento en la producción de citoquinas como interleuquina 1β (IL-1β). AngII e IL-1β estimulan la expresión de enzimas pro-inflamatorias como ciclooxigenasa 2 (COX-2) y NADPH oxidasas, aumentando así la producción de prostanoides y especies reactivas de oxígeno. Entre las isoformas de NADPH oxidasa, NOX-1 y NOX-4 parecen ser particularmente importantes en células musculares lisas vasculares (CMLV). El antígeno humano R (HuR), una proteína de unión a RNA, es capaz de estabilizar diferentes mRNAs favoreciendo el estado pro-inflamatorio en situaciones patológicas. Sin embargo, hasta donde conocemos, ningún estudio ha evaluado si mecanismos post-transcripcionales mediados por HuR participan en el control de la expresión de COX-2 o NOX en CMLV.

El objetivo de esta Tesis Doctoral ha sido investigar los mecanismos transcripcionales y post-transcripcionales implicados en la regulación de la expresión de COX-2, NOX-1 y NOX-4 en CMLV estimuladas con AngII e IL-1β. Adicionalmente, se evaluó el papel de esta regulación en el remodelado vascular. De acuerdo con este objetivo general, la Tesis Doctoral se dividió en dos objetivos específicos que dieron lugar a los siguientes resultados y conclusiones:

1. HuR está implicada en la estabilización del mRNA de COX-2 en células cancerosas y AngII es capaz de estabilizar el mRNA de COX-2 en células vasculares. En la primera parte de esta Tesis Doctoral, analizamos la contribución de HuR en la expresión de COX-2 inducida por AngII e IL-1β y sus consecuencias sobre la migración de CMLV y el remodelado vascular. Los resultados principales fueron: 1) En CMLV, AngII potenció la expresión de COX-2 y tenascina-C (TN-C) y la migración celular inducida por IL-1β. 2) El efecto de AngII sobre la expresión de COX-2 inducida por IL-1β se acompañó de un incremento en la actividad reportera de la 3'UTR y de la estabilidad del mRNA de COX-2 a través de la translocación citoplásmica de HuR y de su unión al

mRNA de COX-2. Estos efectos se bloquearon por inhibidores de ERK1/2 y HuR. 3) La migración de CMLV inducida por AngII+IL-1β se redujo por el bloqueo de COX-2, TXAS, ERK1/2, HuR y los receptores TP y EP y se estimuló por PGE_2 and $TXA_{2.}$ 4) La expresión de COX-2, mPGES-1, TXAS y HuR estaba aumentada en aorta de ratones infundidos con AngII y arterias carótidas ligadas. 5) La expresión de TN-C y el remodelado vascular inducido por la infusión de AngII se abolieron por un inhibidor de COX-2 y por la deleción de mPGES-1. Estos resultados demuestran que la inducción sinérgica de COX-2 producida por AngII+IL-1β en CMLV implica a HuR a través de un mecanismo dependiente de ERK1/2. Nuestros resultados demuestran además, que el eje HuR/COX-2 participa en la migración celular y en el daño vascular.

2. Mecanismos post-transcripcionales dependientes de HuR están implicados en la expresión génica en inflamación; sin embargo, su participación regulando la expresión de NOX no se ha estudiado todavía. En la segunda parte de esta Tesis Doctoral, exploramos los mecanismos transcripcionales y post-transcripcionales implicados en los efectos de AngII e IL-1β en la expresión de NOX-1 y NOX-4 en CMLV y sus implicaciones en la migración celular. Los resultados principales fueron: 1) IL-1β incrementó la expresion de NOX-1, la actividad NADPH oxidasa y la producción de especies reactivas de oxígeno. En cambio, IL-1β disminuyó la expression de NOX-4 y la producción de $H_2O_{2,}$ las cuales no se afectaron por AngII. 2) AngII+IL-1β interfirió con la disminución del mRNA de NOX-1 y promovió la unión de HuR al mRNA de NOX-1. Además, el bloqueo de HuR redujo la estabilidad y los niveles del mRNA de NOX-1 inducidos por AngII+IL-1β. 3) El bloqueo de NOX-1 y de HuR redujo la actividad NADPH oxidasa y la producción de especies reactivas de oxígeno inducidas por AngII+IL-1β. 4) La disminución de la expresión de NOX-4 inducida por IL-1β se bloqueó por un inhibidor de la síntesis proteica y se acompañó de una disminución en la actividad del promotor proximal de 200 bp. 5) AngII y/o IL-1β aumentaron la migración de VSMC que se previno por el bloqueo de NOX-1 y HuR y se aumentó por la sobreexpresión de NOX-4. Así, la estabilización del mRNA de

NOX-1 mediada por HuR es parcialmente responsable de la expresión de NOX-1 y de la producción de especies reactivas de oxígeno en respuesta a AngII+IL-1β en CMLV. Asimismo, mecanismos transcripcionales están implicados en la disminución de la expresión de NOX-4 inducida por IL-1β. La regulación de NOX-1/4 contribuye a la migración de CMLV, proceso importante en condiciones inflamatorias.

En resumen, en esta Tesis Doctoral presentamos evidencias de que la combinación de AngII e IL-1β, dos estímulos inflamatorios importantes en enfermedades cardiovasculares, inducen sinérgicamente la expresión de dos de las principales proteínas pro-inflamatorias COX-2 y NOX-1, las cuales a su vez, participan en la migración de CMLV y en el remodelado vascular. También demostramos por primera vez, que la proteína de unión a RNA HuR es clave en el aumento de la estabilidad del mRNA de ambos genes. Creemos que las rutas HuR/COX-2/NOX-1 serían unas nuevas dianas farmacológicas para prevenir o revertir el remodelado vascular asociado a enfermedades cardiovasculares.

Introduction

Inflammation is the response of the body to injury or infection in order to restore the physiological function and the structure of a tissue. Inflammation has been implicated in the pathogenesis of different diseases such as arthritis, cancer and stroke. In addition, in the last years, it has become evident that inflammation is a key player in cardiovascular disease (Ricciotti and FitzGerald, 2011). During inflammation, the synthesis of different cytokines modulates the expression of adhesion molecules, chemokines and different pro-inflammatory enzymes such as cyclooxygenase-2 (COX-2) or NADPH oxidases (Sprague and Khalil, 2009). These cytokines can act in conjunction with humoral or hormonal mediators important for cardiovascular diseases, such as angiotensin II (AngII), to enhance inflammatory damage (Tummala et al., 1999). COX-2-derived prostanoids or NADPH oxidase-derived reactive oxygen species (ROS) contribute to vascular diseases not only by their direct effects in the vessels but also by enhancing the inflammatory response (Harrison et al., 2011; Ricciotti and FitzGerald, 2011).

In this Introduction, we will review different aspects of the inflammatory process in the context of cardiovascular disease. AngII and interleukin-1β (IL-1β) will be introduced as pro-inflammatory molecules and COX-2 and NADPH oxidase will be presented as key pro-inflammatory enzymes. Finally, the role of COX-2-derived prostanoids and NADPH oxidase-derived ROS in vascular damage will be discussed.

1. VASCULAR REMODELING

1.1. ARTERY STRUCTURE

Arteries are divided in three concentrical layers from the inside out: intima, media and adventitia which are organized in cellular components and extracellular matrix (ECM) (Figure 1).

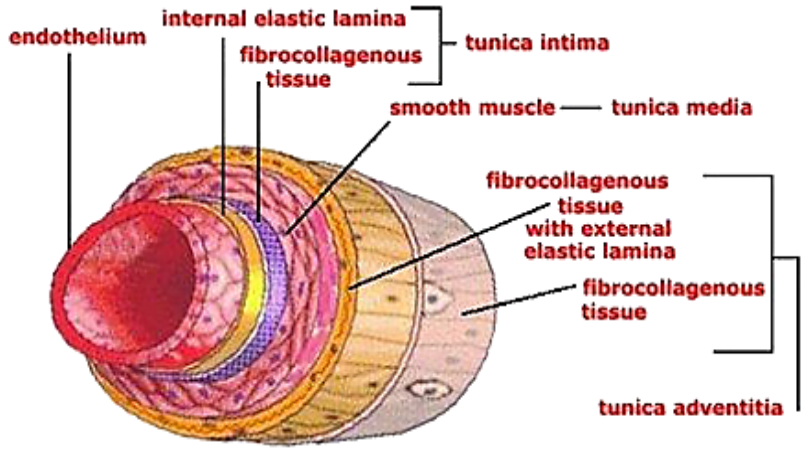

Figure 1. Artery structure. *Wall section showing all layers of an artery wall. From Giannoukos and Min, 2013.*

- Intima: it is in the inner part of the vessel and comprises a monolayer of endothelial cells which lay in the basement membrane. This layer of endothelial cells is separated from the media layer by the internal elastic lamina which is a fenestrated lamina of elastic fibers. The intima layer is important in the control of vascular function and structure because endothelial cells are an important source of vasoconstrictor/vasodilator and proliferative/antiproliferative factors.

- Media: this layer includes circumferentially arranged vascular smooth muscle cells (VSMC) and variable amounts of ECM. The tunica media is separated from the tunica adventitia by a second layer of elastic fibers, the external elastic lamina. In response to different vasoactive factors and hemodynamic forces, VSMC can release a variety of substances which affect vascular tone and structure.

- Adventitia: it is mainly formed by fibroblasts but it also contains macrophages and mast cells and different components of the ECM. In the last years, it has become evident that the adventitia is not only a mechanical support for the vessel but also an active player of the regulation of vascular tone and structure by releasing different factors.

The ECM is a gel-like form which functions as a scaffolding structure for the vascular cells and determines the elasticity and mechanical properties of the vessels. Their components are synthetized by different cell types of the vascular

wall. The two main ECM proteins are collagen and elastin; while elastin confers the elastic properties to vessels, collagen provides the strength (Wagenseil et al., 2009). There are other ECM proteins which are in less quantity such as glycoproteins, proteoglycans and integrins that are involved in several cellular processes (Wagenseil et al., 2009). Among them, tenascin-C (TN-C) which is an inducible glycoprotein expressed predominantly in embryonic, remodeled adult tissues and in pathological conditions, is particularly interesting. Competitive binding of TN-C to ECM proteins and their counterpart cell-surface receptors mediates its ability to modulate cell-ECM interactions. The capacity of TN-C to interact with a wide range of ECM molecules may also enable it to contribute to the structural organization of the ECM. In addition, TN-C can promote migration and proliferation by direct activation of cell-surface growth factor receptors and cellular differentiation by up-regulating androgen receptor and endothelin type 1 receptor expression (Milwood and Orend, 2009). Thus, TN-C relevance relies on its implication in vascular cell differentiation, proliferation and migration (Milwood and Orend, 2009).

1.2. TYPES OF VASCULAR REMODELING

It is now accepted that the vascular wall can change its structure in order to maintain the appropriate lumen size to permit normal blood flow. This process is termed vascular remodeling (Gibbons and Dzau, 1994). This ability of the arteries to adapt its structure in response to physiological and pathological conditions is essential in situations such as pregnancy or aging but also in many arterial diseases. Thus, the inability of the vessels to remodel appropriately is considered a form of “vascular failure” that can lead to pathologic states such as hypertension, atherosclerosis or restenosis (Renna et al., 2013). This process is active and involves structural changes including cell growth, death, migration and the synthesis or degradation of the ECM (Renna et al., 2013).

Vascular remodeling can occur with or without growth of the vessel wall (i.e. hypertrophic, eutrophic or hypotrophic) and with smaller, greater or similar lumen size (inward, outward or compensated) (Figure 2) (Mulvany et al., 1999;

Varik et al., 2012). Vascular remodeling differs depending on the vessel type or the cardiovascular disease model:

- *Hypertrophic remodeling* is characterized by an increase in the media thickness, media/lumen and vascular cross sectional area associated with a more evident contribution of cell growth (Mulvany, 1999) (Figure 2). This type of remodeling is characteristic of large arteries in ageing or in pathologies like hypertension (Schiffrin, 2012) or restenosis which is associated with proliferation and migration of different cells types (Renna et al., 2013).

- *Hypotrophic remodeling* is associated with a decrease in the amount of material (i.e. diminished cross sectional area) around the vessel wall (Mulvany, 1999) (Figure 2). This type of remodeling may be related to apoptosis processes and/or to rearrangement of the material in the vessel wall (Intengan and Schiffrin, 2001). Hypotrophic remodeling has been shown in renal afferent arterioles from spontaneously hypertensive rats (SHR) (Nørrelund et al., 1994) and in mesenteric resistance arteries from ouabain-induced hypertensive animals (Briones et al., 2006).

- *Eutrophic remodeling* is characterized by a decrease in the outer and lumen diameters and an increase in the media thickness and the media/lumen ratio with no change in the wall cross sectional area (Mulvany, 1999) (Figure 2). It has been suggested that this type of remodeling is due to rearrangement of the same amount of wall material around a smaller vessel lumen (Heagerty et al., 1993; Rizzoni and Agabiti-Rosei, 2012). The mechanisms leading to this type of remodeling are poorly known but some authors suggest that a combination of inward growth and peripheral apoptosis or prolonged vasoconstriction of vascular cells embedded in an expanded ECM can lead to eutrophic remodeling (Bakker et al., 2002; Schiffrin, 2012).

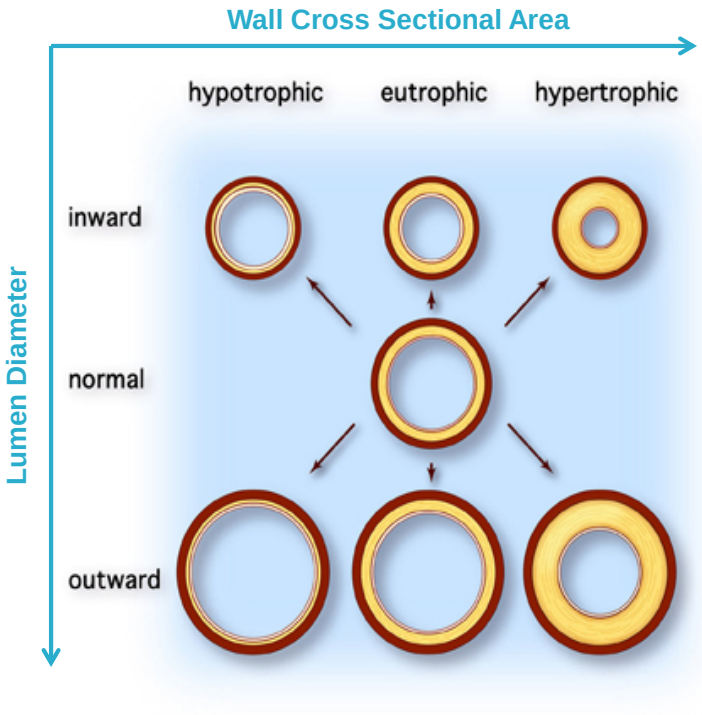

Figure 2. Types of vascular remodeling. *Classification refers to changes on the lumen diameter (inward: upper row or outward: lower row) and vessel cross-sectional area (hypotrophic: left column; eutrophic: center column and hypertrophic: right column). Adapted from van Varik et al., 2012.*

Vascular remodeling can be induced by dynamic interactions between local growth factors, vasoactive substances and hemodynamic stimuli being all important mediators in the vascular adaptation process. The number of mediators involved in altered vascular structure is continuously growing; however, to date it is well admitted that AngII, cytokines, prostanoids and ROS have a key role (Renna et al., 2013). The participation of these mediators in vascular remodeling will be discussed in subsequent sections of this Introduction.

1.3. CELL PROLIFERATION AND MIGRATION

As mentioned, wall thickening is one of the main features of many cardiovascular diseases. Depending on the specific vascular bed and pathology, the cellular and non-cellular events leading to altered vascular structure might be different. Thus, hypertension causes arterial media thickening with or without cell growth, and ECM deposition in both humans and animal experimental models; however, atherosclerosis and reaction to injury

such as endothelial denudation or restenosis cause intimal thickening associated to variable degrees of alterations in the surrounding ECM. Although it is known that during vascular remodeling VSMC proliferation and migration are processes that take place, their regulation is not exactly known (Moore, 2013) (Figure 3).

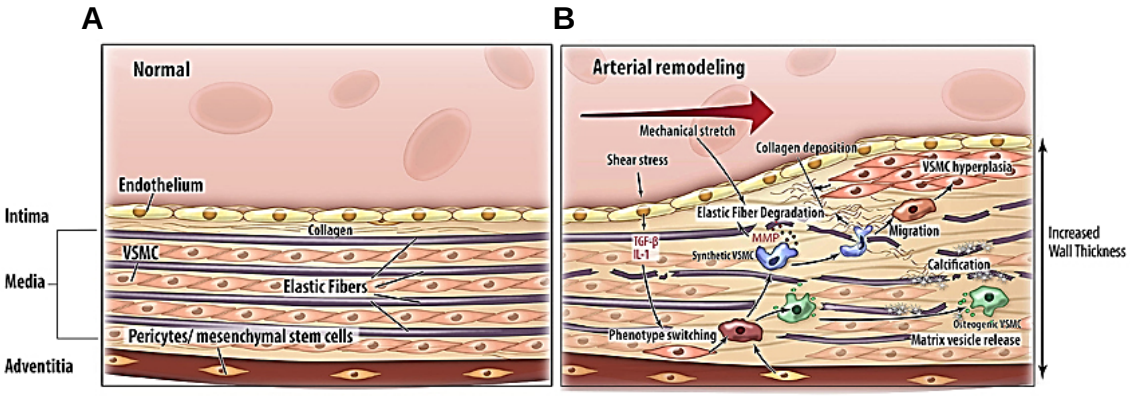

Figure 3. Pathophysiological mechanisms of arterial remodeling. *Cross sectional schematic view of the arterial wall in* ***(A)*** *normal situation or* ***(B)*** *during arterial remodeling. Thickening of the wall is the main feature of arterial remodeling. Elastic fiber degradation, extracellular matrix calcification, collagen deposition and vascular smooth muscle cell migration and phenotype switching lead to adaptation of the vascular wall. Matrix metalloproteinase (MMP). Taken from van Varik et al. 2012.*

Intimal thickening can occur in blood vessels as a consequence of physiological process as occurs in ageing or in response to increased intraluminal pressure, or in response to injury as observed in balloon dilatation, stent implantation or atherosclerosis processes (Newby, 2006). Because of its importance, many *in vivo* models of VSMC growth and proliferation such as the carotid ligation mouse model have been developed. In this model, an intima lesion characterized by enrichment of VSMC occurs in response to luminal narrowing leading to the formation of the neointima (Newby, 2006; Moore, 2013). Neointima is part of the reparative response to injury and its formation involves an important inflammatory component with infiltration of inflammatory cells and release of cytokines and chemokines, thrombosis, increase in the number of VSMC and matrix production leading to a reduction in vessel diameter (Kumar and Lindner, 1997; Kawasaki et al., 2001; Moore, 2013). The increased number of VSMC is mainly originated by migration from the underlying media and proliferation, although there are other processes involved

such as transdifferentiation of endothelial cells or differentiation from circulating precursors (Newby, 2006; Renna et al., 2013) (Figure 3).

The involvement of cell proliferation and/or migration in hypertensive vascular remodeling mainly depends of the vascular bed and the experimental model studied. Thus, coronary but not mesenteric vessels from SHR show increased VSMC number (Roque et al., 2013). In addition, administration of AngII, the main effector peptide of the renin-angiotensin-aldosterone system (RAAS) lead to a progressive increase in blood pressure and media thickening through migration, proliferation and hypertrophy of the VSMC, being this effect mediated through the AngII type 1 receptor (AT_1R) (Xu and Touyz, 2006; Valente et al., 2012; Ozasa et al., 2013; Renna et al., 2013) (Figure 4). Importantly, in the last years it has become evident that vascular infiltration of immune inflammatory cells and pro-inflammatory mediators such as ROS are key contributors to the vascular remodeling observed in this pathology (Bush et al., 2000; De Ciuceis et al., 2005; Harrison, 2014).

Cell proliferation and migration begin with stimulation of cell surface receptors that transduce the external signal to a series of coordinated responses inside the cell. Diverse signal transduction systems such as nuclear factor kappa B (NF-kB), the activator protein 1 (AP-1), the mitogen activated protein kinases (MAPKs) or the phosphatidylinositol-3-kinase (PI3K)/Akt pathways have been proposed to translate the stimulus within VSMC (Rudijanto et al., 2007). However, despite of the growing information regarding the mechanisms controlling VSMC migration and proliferation in response to stimuli such as AngII (Figure 4), their regulation in response to other stimuli is less known.

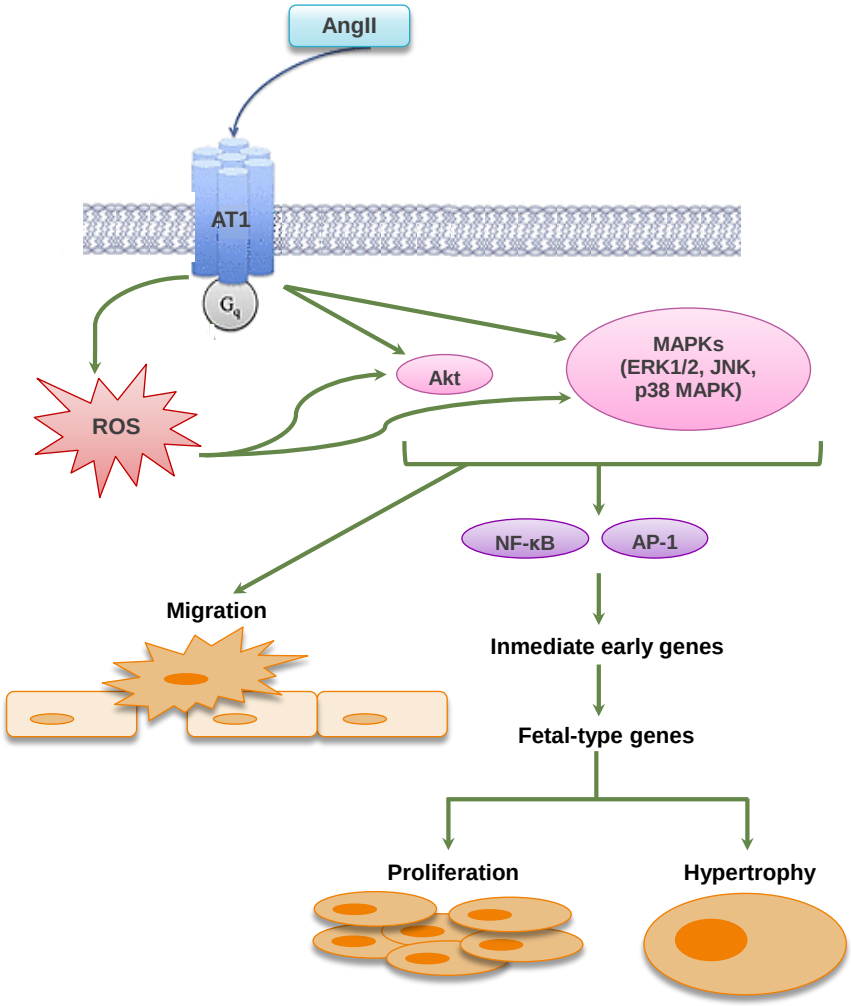

***Figure 4. AngII induces proliferation, migration and/or hypertrophy of VSMC**. Arrows indicate the main biological end points preceding cell proliferation, migration and hypertrophy in response to AngII. Adapted from Chiou et al., 2011.*

2. INFLAMMATION

Growing evidences suggest that an imbalance between pro- and anti-inflammatory mediators is a common pathophysiological mechanism in different cardiovascular diseases (Kofler et al., 2005; Vicenová et al., 2009). Inflammation often begins with endothelial activation through several signal transduction mechanisms, leading to the expression of adhesion molecules which attracts different immune cells (Libby, 2006). These immune cells, as well as the resident cells participate as donors and recipients of cytokine signals, amplifying the inflammatory activity (Kofler et al., 2005; Libby, 2006). It is now accepted that classical mediators of vascular damage such as different components of the RAAS including AngII and aldosterone induce pro-

inflammatory actions in different cell types of the vascular wall (Sprague and Khalil, 2009; Brown, 2013). This inflammatory environment has deleterious consequences in different functions of the vasculature including contraction/dilation, stiffness and vascular structure (Chen et al., 2013a; 2014). In this section, we will focus on different molecules with potential pro-inflammatory properties at vascular level and on two pro-inflammatory proteins, COX-2 and NADPH oxidase. We will also highlight findings linking these mediators and vascular remodeling.

2.1. INFLAMMATORY MEDIATORS OF VASCULAR DAMAGE

2.1.1. Cytokines

Cytokines are a broad category of small soluble proteins including chemokines, interferons, ILs, lymphokines tumor necrosis factors (TNFs) and transforming growth factors (TGFs) which are important in cell signaling and are produced not only by immune cells like macrophages and B and T lymphocytes, but also by endothelial cells, VSMC or fibroblasts. Both anti- and pro-inflammatory cytokines have been described. Anti-inflammatory cytokines include TGF-β, IL-4, IL-10 or IL-13 and the main pro-inflammatory cytokines are TNF-α, IL-1 or IL-6. As mentioned, the balance between anti- and pro-inflammatory cytokines is shifted to pro-inflammatory cytokines in cardiovascular disease (Kofler et al., 2005; Vicenová et al., 2009) (Figure 5).

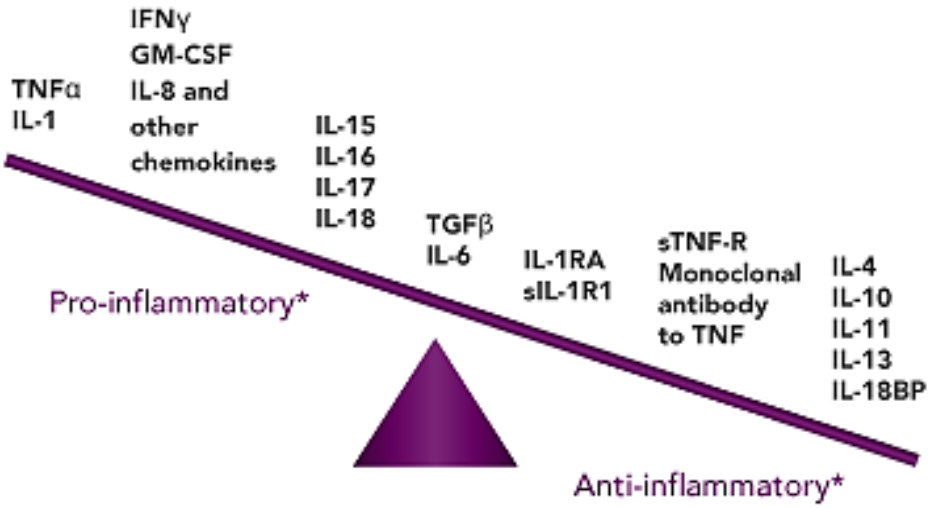

Figure 5. Anti- and pro-inflammatory cytokines. *Implication of cytokines in inflammation. Granulocyte macrophage colony-stimulating factor (GM-CSF); IL-18BP (IL-18-binding protein); sIL-1R1 (Soluble IL-1R I); sTNF-R (Soluble TNF receptor). Taken from Arend, 2001.*

IL-1 family is a key player in autoimmune disorders and has been recently demonstrated to be involved in the development of several cardiovascular diseases (Vicenová et al., 2009). This family is composed by eleven members where IL-1α and IL-1β are the best known. IL-1β is synthesized as a precursor and is produced after stress or cell injury by macrophages and other immune or non-immune cells. The secretion of IL-1β needs two steps, the induction of its synthesis and a proteolytic cleavage. The main targets of IL-1β are cells of the immune system such as monocytes or lymphocytes, but these cytokines can also act on other cells such as fibroblasts, endothelial cells or VSMC to induce inflammatory responses (Vicenová et al., 2009). The major receptor that mediates the effects of IL-1β is the type I IL-1 receptor (IL-1RI) which needs IL-1 receptor accessory protein (IL-1RAcP) as a co-receptor for signal transduction. After activation, IL-1RI interacts through its Toll/interleukin-1 receptor (TIR) domains with the TIR domain of the signaling adaptor myeloid differentiation factor 88 (MyD88), which transduces the signal to a family of IL-1R-associated kinases (IRAKs). Phosphorylation of IRAK by other IRAK family members induces cascades of signaling through TNF receptor-associated factor 6 (TRAF6) that will facilitate the association of TGF-β-activated protein kinase 1 (TAK1) with TRAF6. The activation of both IKK and MAPKs by TRAF6

appears to involve the TAK1 and MAPK kinase kinase (MAPKKK) respectively. On the other hand, PI3K activation can occur through TRAF6 and receptor interacting protein (RIP). The stimulation of these signaling pathways lead to activation of different transcription factors such as NF-κB, AP-1 which are involved in the synthesis of pro-inflammatory genes including COX-2 or NADPH oxidase-1 (NOX-1) (O'Neill and Greene, 1998; Sizemore et al., 1999; Martín et al., 2003; Vicenová et al., 2009; Weber et al., 2010) (Figure 6).

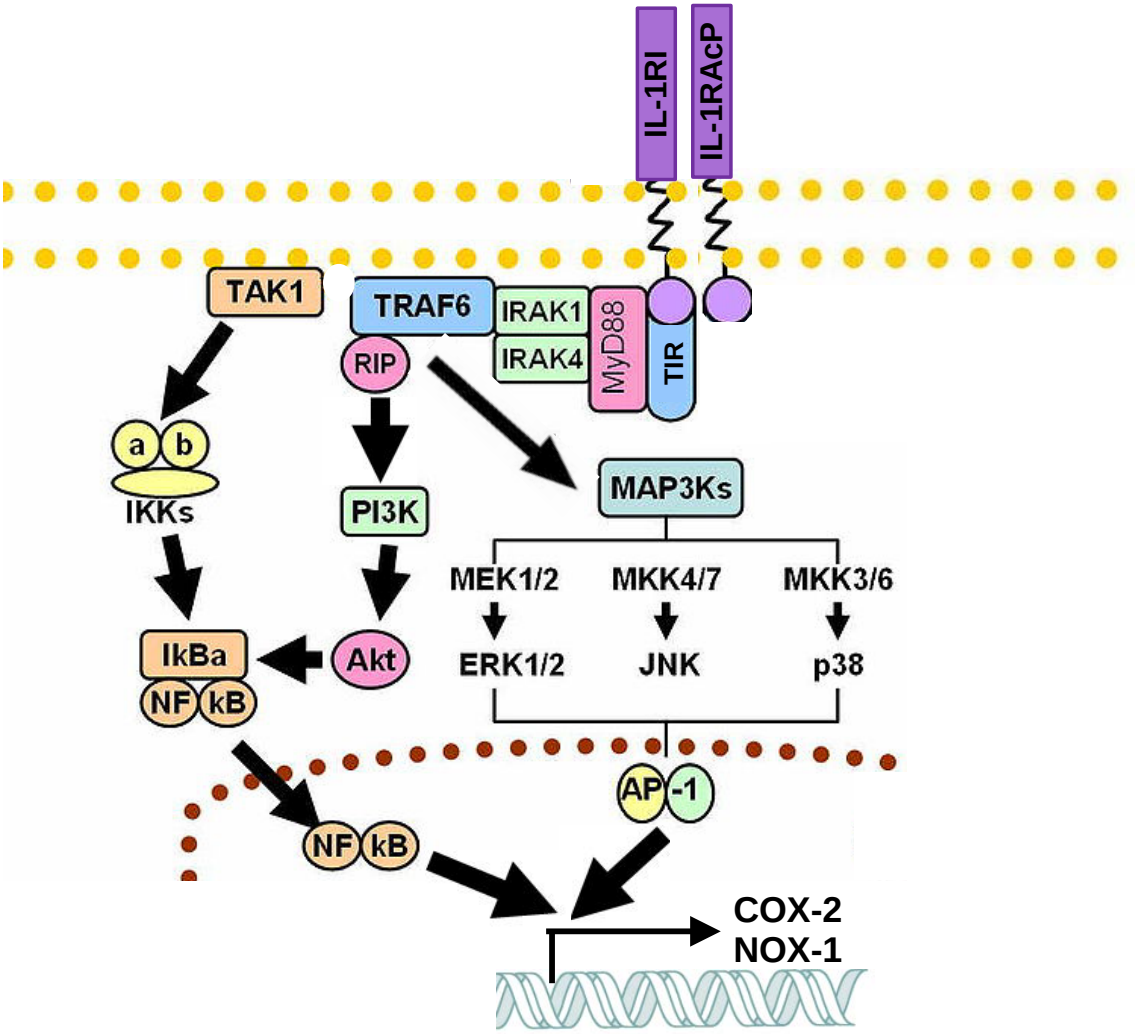

Figure 6. Schematic illustration of IL-1 receptor signaling. *In response to activation by IL-1, IL-1RI interacts with different proteins leading to the activation of NF-κB, MAPKs and PI3K/Akt leading to the synthesis of pro-inflammatory cytokines and enzymes. Inhibitor of nuclear factor kappa-B (IκB); Inhibitor of nuclear factor kappa-B kinase (IKK); MAPK kinase kinase (MAP3K); Mitogen-activated protein kinase kinase (MEK, MKK); Receptor interacting protein (RIP); Toll/interleukin-1 receptor (TIR) domain. Modified from McCulloch et al., 2006.*

MAPKs are one of the most important signaling pathways involved in cell responses and are activated by IL-1β. MAPKs include different cascades being extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 MAPK the best known. Each cascade is initiated by the serine/threonine phosphorylation of a MAPKKK, which phosphorylates tyrosine/threonine residues of MAPK kinases (MAPKK), and subsequently leads to phosphorylation and activation of MAPKs. The activated MAPKs then activate other protein kinases, nuclear proteins and transcription factors, leading to various cellular responses. Thus, while ERK1/2 is more related to cell proliferation, JNK and p38 MAPK are more involved in different processes such as cell cycle arrest, inflammation and apoptosis (Pearson et al., 2001) (Figure 7).

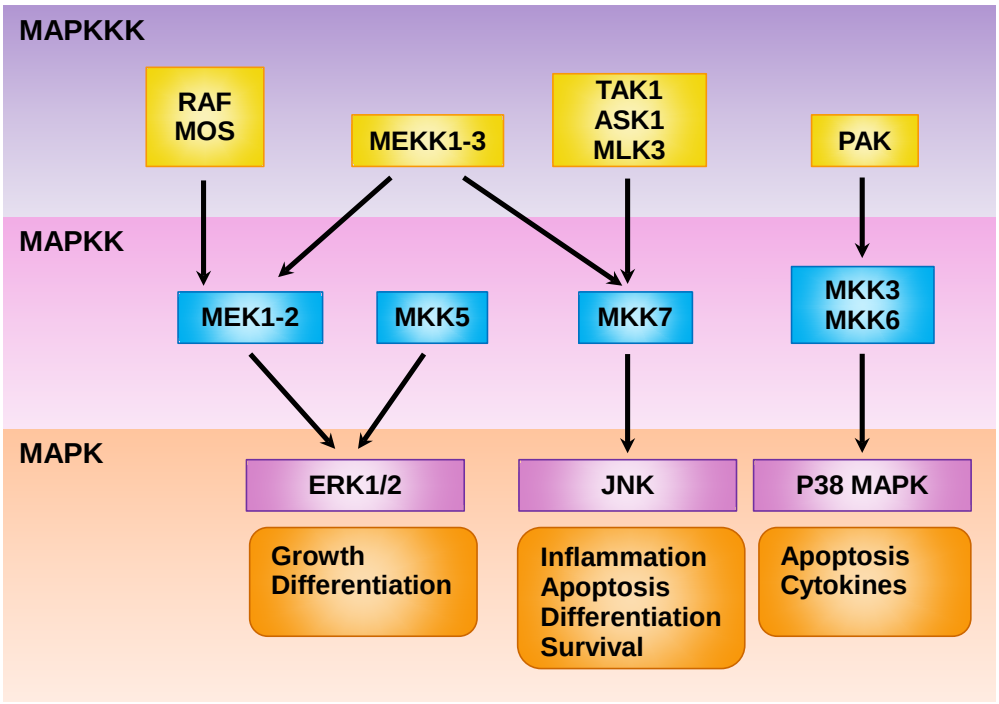

Figure 7. The three arms of the mitogen-activated protein kinase (MAPK) signaling pathway: ERK1/2, JNK and p38 MAPK. *Each arm is initiated by the phosphorylation of a MAPK kinase kinase (MAPKKK), which phosphorylates a MAPK kinase (MAPKK) and subsequently leads to phosphorylation and activation of a MAPK involved in different cellular responses. Apoptosis signal-regulating kinase (ASK); MAPK kinase kinase (MEKK); MAPK kinase (MEK, MKK); Mixed-lineage protein kinase 3 (MLK); V-mos Moloney murine sarcoma viral oncogene homolog (MOS); P21 protein (Cdc42/Rac)-activated kinase 1 (PAK); Rapidly accelerated fibrosarcoma (RAF); TGF-β-activated kinase 1 (TAK). Adapted from Steeg, 2003.*

PI3K is another signaling pathway activated by IL-1β which is involved in different cellular processes. The PI3Ks are members of a unique and conserved family of intracellular lipid kinases that phosphorylate phosphatidylinositol and phosphoinositides. There are three classes (I–III) implicated in many functions such as cell metabolism, survival and polarity. The activation of a receptor protein tyrosine kinase (RPTK) results in autophosphorylation of tyrosine residues. Phosphatidylinositol-3 kinase (PI3K) consisting of an adaptor subunit p85 and a catalytic subunit p110 is translocated to the cell membrane and binds to phosphotyrosine residues of the RPTK. This results in activation of PI3K leading to production of phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 recruits Akt with the pleckstrin homology (PH) domain to the cell membrane, promoting T308 and S473 residue phosphorylation by protein serine/threonine kinase 3'-phosphoinositide-dependent kinases 1 and 2 (PDK1 and PDK2) necessary for maximal Akt activation. Activated Akt translocates to the nucleus and mediates the inhibition of Bcl-2-associated death promoter (Bad) and activation of mammalian target of rapamycin (mTOR), among others, which are involved in apoptosis and cell survival respectively, thus promoting cell survival, growth and proliferation. Phosphatase and tensin homologue deleted from chromosome 10 (PTEN) is a PIP_3 phosphatase and negatively regulates the PI3K/Akt pathway (Engelman et al., 2006) (Figure 8).

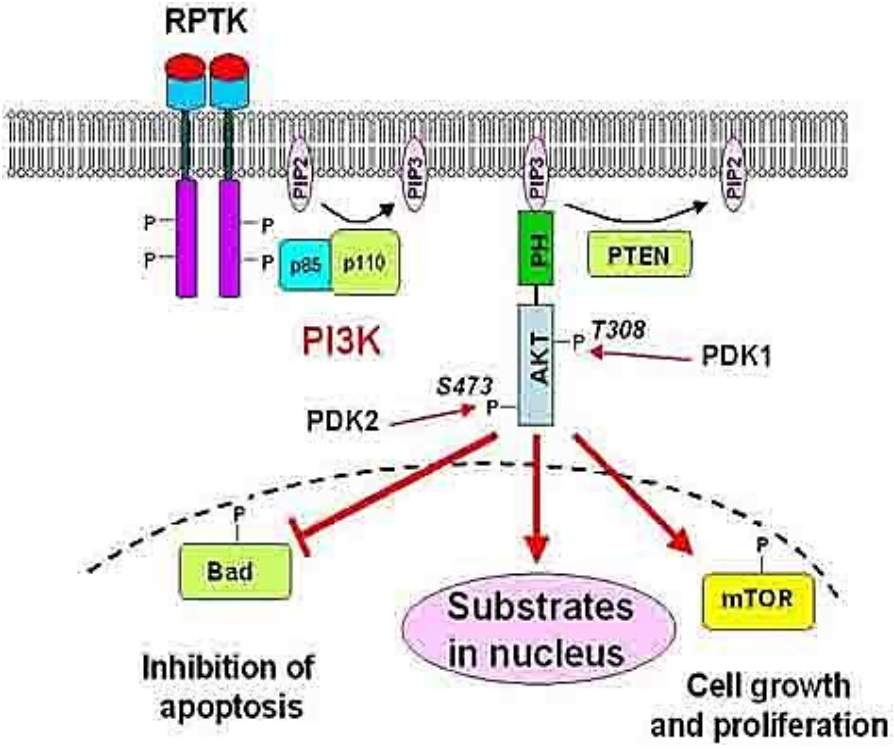

Figure 8. Schematic representation of the PI3K/AKT signaling pathway. *Activation of a receptor protein tyrosine kinase (RPTK) results in PI3K translocation to the cell membrane and activation leading to Akt activation and translocation to the nucleus regulating different proteins. Bcl-2-associated death promoter (Bad); Mammalian target of rapamycin (mTOR); Protein serine/threonine kinase 3'-phosphoinositide-dependent kinases (PDK); phosphatase and tensin homologue deleted from chromosome 10 (PTEN); Phosphatidylinositol phosphate (PIP). Taken from Meier et al., 2005.*

Role of cytokines in inflammation and vascular remodeling

The importance of cytokines on vascular diseases relies on the fact that they are increased systemically or locally in different cardiovascular diseases. Thus, serum samples from untreated hypertensive patients (Dalekos et al., 1997) and human coronary arteries from patients suffering coronary atherosclerosis or cardiomyopathy (Galea et al., 1996) show elevated levels of IL-1β. More importantly, in some cardiovascular diseases, IL-1β levels correlates with disease severity (Galea et al., 1996).

The two main mechanisms responsible for IL-1β-induced pro-inflammatory actions are recruitment of immune cell infiltration and synthesis of pro-inflammatory enzymes. The role of IL-1β inducing vascular pro-inflammatory proteins such as COX-2 and NADPH oxidase will be reviewed in subsequent

sections of this Introduction. Regarding immune cell infiltration, it has been described that deletion of the natural inhibitor of IL-1, IL-1R antagonist (IL-1Ra), further impaired vascular damage induced by apolipoprotein E (ApoE) deletion leading to a severe form of aortic inflammation with massive infiltration of macrophages in the adventitia (Merhi-Soussi et al., 2005). In addition, in the absence of IL-1β, the severity of atherosclerosis in ApoE$^{-/-}$ mice decreases possibly through the decreased expressions of vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemotactic protein (MCP-1) in the aorta (Kirii et al., 2003). The inflammatory reaction occurring in the vascular wall has deleterious functional and structural consequences. Specifically, IL-1β is directly involved in vascular remodeling as demonstrated by the fact that overexpression of IL-1Ra can inhibit the development of atherosclerotic lesions in ApoE$^{-/-}$ mice (Merhi-Soussi et al., 2005). In the carotid ligation mouse model, IL-1R$^{-/-}$ mice show attenuated neointimal hyperplasia (Rectenwald et al., 2000). Accordingly, *in vitro* experiments show that IL-1β induces VSMC proliferation and migration (Kim et al., 2010; Wang et al., 2011a). Altogether, these results indicate that IL-1 plays a critical role in the pathogenesis of cardiovascular disease and in vascular remodeling.

2.1.2. Angiotensin II

AngII is the main hormone peptide of the RAAS. RAAS is activated when blood pressure is low. First step is the renin secretion to blood mediated by juxtaglomerular cells. Plasma renin converts angiotensinogen released by the liver to AngI. Then, AngI is transformed to AngII by the angiotensin-converting enzyme (ACE). AngII causes constriction of blood vessels leading to a blood pressure increase and augments aldosterone secretion from the adrenal cortex. Aldosterone is a hormone that increases sodium and water reabsorption in the tubules of the kidneys thus increasing blood volume and therefore blood pressure (Mehta and Griendling, 2007) (Figure 9).

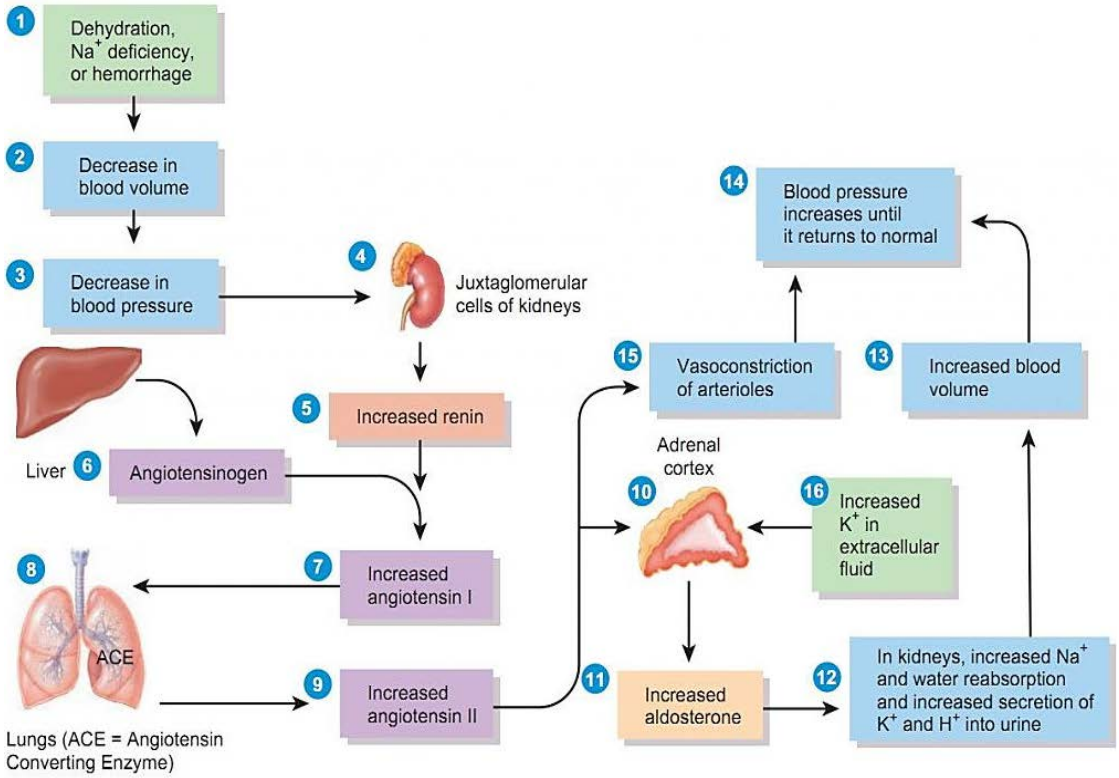

Figure 9. Renin-Angiotensin-Aldosterone system. *Steps of the secretion of the renin and its conversion in AngII leading to an increase in the blood pressure. Taken from http://antranik.org/wp-content/uploads/2012/05/renin-angiotensin-aldosterone-reflex-system.jpg?783ed6.*

AngII effects are mediated by different receptors, being the two major AngII receptors AT_1 (AT_1R) and AT_2 (AT_2R). AT_1R and AT_2R are G protein-coupled receptors (GPCRs); however, they only share 30% of homology in the amino acid sequence (Rüster and Wolf, 2006). AT_1R is highly expressed in cardiovascular cells, such as VSMC and it is coupled to $G_{\alpha q/11}$, as well as $G_{\alpha i}$, $G_{\alpha 12/13}$. Apart from its effects on vascular contraction and aldosterone secretion, AT_1R signaling lead to pro-inflammatory, proliferative and anti-apoptotic processes which explain at least in part, its effects in cardiovascular diseases. Signaling of AT_1R activates mitogenic signaling pathways including MAPKs or PI3K, either directly or indirectly for example, through GPCR-mediated transactivation of the epidermal growth factor receptor (EGFR), or through other mechanisms including Cell division control protein 42 homolog (CDC42)/JNK or p38 MAPK signaling. This leads to the downstream activation of transcription factors, including NF-κB which is responsible for the generation of growth factors, cytokines, ECM and anti-apoptotic proteins (Figure 10). On the other

hand, AngII can promote apoptosis through AT_2R by mechanisms linked to the inhibition of ERK1/2 signaling and the activation of cell death receptors (Nouet and Nahmias, 2000; George et al., 2010) (Figure 10).

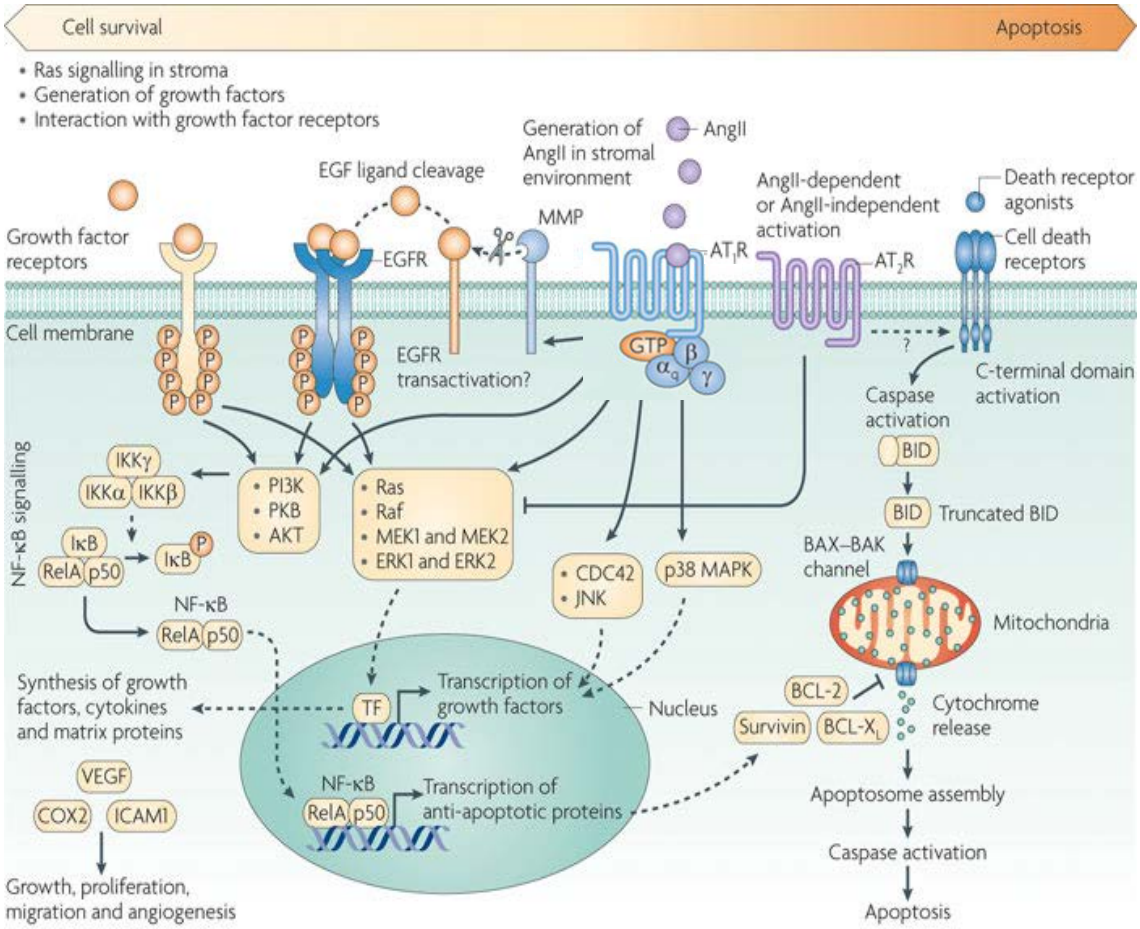

Figure 10. AngII signaling and implications in cell survival. *AT_1R signaling activates MAPKs or PI3K, activating transcription factors including NF-κB. In contrast AT_2R signaling has been linked to the activation of cell death receptors, and thus, apoptosis. B-cell lymphoma (BCL); Bcl2 interacting protein (BID); Cell division control protein 42 homolog (CDC42); Inhibitor of nuclear factor kappa-B (IκB); Inhibitor of nuclear factor kappa-B kinase (IKK); Mitogen-activated protein kinase kinase (MEK); Matrix metalloproteinase (MMP); Protein kinase B (PKB); Rapidly accelerated fibrosarcoma (Raf); Transcription factor (TF). Taken from George et al., 2010.*

Role of angiotensin II in inflammation and vascular remodeling

It is well accepted that chronically elevated levels of AngII play a key role in hypertension, atherosclerosis and restenosis (Mehta and Griendling, 2007). AngII is able to generate an inflammatory environment associated to the up-regulation of adhesion molecules and production of chemokines which lead to monocytes/macrophages recruitment to the vascular wall (Intengan and

Schiffrin, 2001). This inflammatory response is observed *in vivo* in the AngII infusion model where increased expression of adhesion molecules such as endothelial-selectin (E-selectin), ICAM-1 and VCAM-1 is observed (Das, 2005; Xu et al., 2011). In this model, pro-inflammatory cytokines including TNF-α, IL-6 and MCP-1 expressions are increased as well as macrophage infiltration in the arterial wall (Bush et al., 2000; Liu et al., 2003; Das, 2005; Xu et al., 2011). In addition, AngII stimulates the expression of key pro-inflammatory enzymes such as COX-2 and NADPH oxidase at vascular level (Virdis et al., 2011; Hernanz et al., 2014). This aspect will be reviewed in subsequent sections of this Introduction.

AngII influences the architecture and integrity of the vascular wall by modulating cell growth and regulating ECM composition (Schiffrin, 2002; Virdis et al., 2013). Thus, either *in vitro* or *in vivo*, AngII induces VSMC growth, migration or hypertrophy, and induces changes in different ECM proteins (Briones et al., 2010; Mugabe et al., 2010; Lacolley et al., 2012). Proof of the key role of RAAS in the vascular alterations in hypertension is the fact that chronic administration of ACE inhibitors (ACEI) or AngII receptor blockers (ARBs), lowers blood pressure and improve vascular remodeling in several hypertensive animal models including the SHR model, which is classically associated with hyperactivation of the vascular RAAS (Intengan et al., 1999; Schiffrin, 2012; Briones et al., 2014). More importantly, hypertensive patients treated with ACEI, ARBs or with calcium (Ca^{2+}) channel blockers but not with the β-blocker, atenolol, exhibited significant regression of vascular remodeling of large and small arteries independently of hemodynamic changes (Schiffrin et al., 2000; Schiffrin and Touyz, 2004; Briet and Schiffrin, 2013; Briones et al., 2014). Interestingly, AngII signaling through AT_1R is significantly enhanced in cultured VSMC from resistance arteries of hypertensive rats or patients (Touyz and Schiffrin, 2001; Touyz et al., 2002) which might contribute to the deleterious effects of AngII at vascular level in pathological conditions.

2.2. PRO-INFLAMMATORY PROTEINS

In this section we will discuss different aspects regarding two of the main pro-inflammatory proteins and their respective mediators: COX-2 and

prostanoids, and NADPH oxidase and ROS. Major efforts will be put on highlighting specific aspects of their regulation and their role in vascular remodeling.

2.2.1. Cyclooxygenase-2

Biosynthesis of prostanoids begins with the formation of PGG_2/PGH_2 through the action of COXs on arachidonic acid released by phospholipases from the membrane phosphoglycerides. Different prostaglandin synthases (PGS) metabolize immediately the PGH_2 into specific prostanoids (Figure 11).

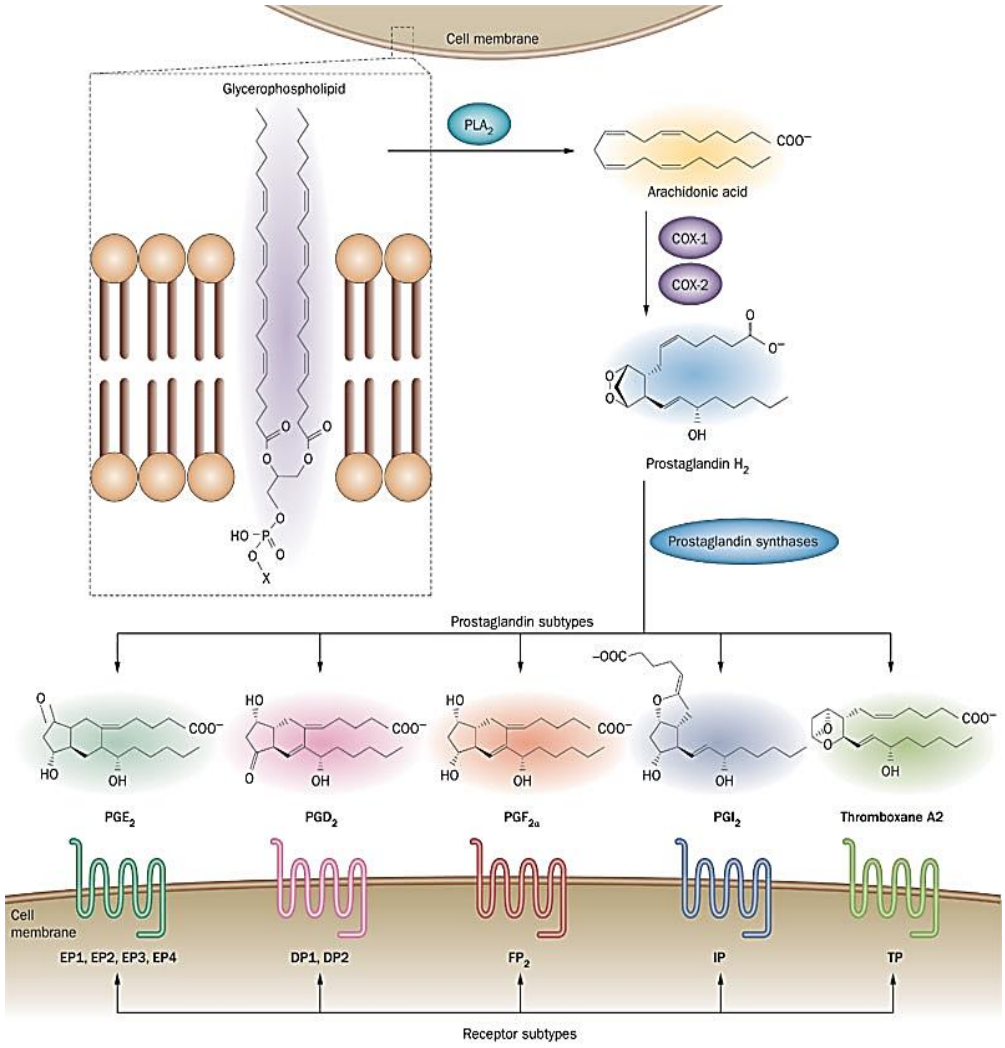

Figure 11. Prostanoid synthesis. *COX-1 and COX-2 enzymes catalyze the biosynthesis of PGH_2, which is subsequently converted into five prostanoids: the prostaglandins, PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2. These prostanoids bind to their own receptors: EP1-4, DP1-2, FP_2, IP and TP, respectively which belong to the GPCRs. Taken from Rahnama'i et al., 2012.*

COXs have two different active sites with different activities. The cyclooxygenase site transforms arachidonic acid into the hydroperoxy endoperoxide prostaglandin G_2 (PGG_2). The other site contains a heme group with a peroxidase activity which catalyzes the reduction of PGG_2 into PGH_2 (Davidge, 2001; Warner and Mitchell, 2004). Three different COX isoforms have been described: COX-1, COX-2 and COX-3, where COX-3 is a splice variant of COX-1, thus, COX-3 is sometimes named as COX-1b or COX-1v (Warner and Mitchell, 2004). In most tissues COX-1 is constitutively expressed under physiological conditions. In fact, COX-1 promoter contains GC rich elements present in housekeeping genes (Tanabe and Tohnai, 2002). However, COX-1 induction mediated by shear stress (Doroudi et al., 2000), IL-1β (Kirtikara et al., 1998) or vascular endothelial growth factor (VEGF; Bryant et al., 1998) has also been described.

COX-2 is an inducible isoenzyme and the dominant source of PGs in inflammation. COX-2 gene is approximately 8.3 kb long with 10 exons and its sequence contains 604 amino acids. It shares a 61% of homology with the human COX-1 protein and its gene structure is similar between different species (Tanabe and Tohnai, 2002). While COX-1 is localized in the endoplasmic reticulum (ER), COX-2 acts predominantly at ER and nuclear envelope (Morita et al., 1995). In vascular cells, COX-2 expression is induced by a wide variety of stimuli such as AngII, IL-1β, ROS, lipopolysaccharide (LPS) or TNF-α (Tanabe and Tohnai, 2002; Kuwano et al., 2004; Galán et al., 2011; Martínez-Revelles et al., 2013) and it is up-regulated in vascular inflammatory diseases such as aortic aneurysms (King et al., 2006) and balloon-injured arteries (Yang et al., 2004a; Wang et al., 2005). In addition, arteries from hypertensive animal models including AngII infusion and SHR, and hypertensive patients show increased vascular COX-2 expression and (Hernanz et al., 2004; Adeagbo et al., 2005; Álvarez et al., 2005; Virdis et al., 2009; Virdis et al., 2013; Martínez-Revelles et al., 2013) and this is normalized by treatment with ARBs (Álvarez et al., 2007).

2.2.1.1. Cyclooxygenase-2 regulation

COX-2 is an immediate-early response gene and its expression can be regulated by transcriptional and post-transcriptional mechanisms such as mRNA stability (Tanabe and Tohnai, 2002).

COX-2 promoter contains a TATA box and several binding sites for transcription factors that are responsible for its expression. Thus, NF-κB, AP-1, cyclic AMP-responsive binding protein (CREB) (Chun and Surh, 2004), CCAAT-enhancer-binding proteins (C/EBP) (Iñiguez et al, 2000), V-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS-1) (Grall et al., 2005), T-cell factor/lymphoid enhancer factor-response element (TCF/LEF) (Nuñez et al., 2011) or human epidermal growth factor receptor 2 (ErbB-2) (Wang et al., 2004) bind to the COX-2 promoter in response to different stimuli (Figure 12).

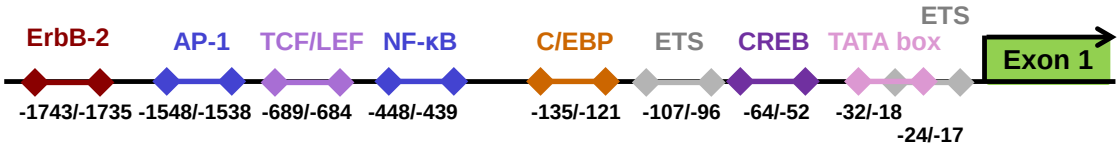

Figure 12. Structure of human COX-2 gene promoter. *Binding sites for different transcription factors involved in COX-2 expression. CCAAT-enhancer-binding proteins (C/EBP); V-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS-1); T-cell factor/lymphoid enhancer factor-response element (TCF/LEF); human epidermal growth factor receptor 2 (ErbB-2).*

Depending on the stimulus and the cell type, several signaling pathways can participate in COX-2 expression (Tsatsanis et al., 2006). For example, ERK1/2 and p38 MAPK (Ohnaka et al., 2000) pathways are involved in AngII-induced COX-2 expression in VSMC. Similarly, in vascular fibroblasts, IL-1β and the combination of AngII plus IL-1β stimulate COX-2 expression through ERK1/2 and p38 MAPK (Galán et al., 2011).

Besides transcriptional regulation, COX-2 expression seems to be under the control of the post-transcriptional 3'untranslated region (3'UTR) regulation carried out by miRNA and RNA-binding proteins (RBPs) (Cok et al., 2003; Sureban et al., 2007; Young et al., 2012). A key feature present in the COX-2 3'UTR is the adenylate and uridylate (AU)-rich element or ARE (Dixon et al., 2000). These elements control mRNA turnover, translation and/or transport by

interacting with RBPs. Most of the RBPs have been associated with mRNA degradation [e.g., AU-rich element RNA-binding protein 1 (AUF1), RNA polymerase III transcription initiation factor 90 KDa subunit (BRF1) or tristetraprolin (TTP)] and/or suppression of translation (e.g., T-cell intracellular antigen 1 (TIA-1), TIA-1 related protein (TIAR)] (Meisner and Filipowicz, 2011). In contrast, the RBPs which belong to Hu family induce ARE-mediated mRNA stabilization. One of these members is the HuR protein (Hu antigen R; ELAVL1) that can stabilize different mRNAs, including COX-2 mRNA (Dixon et al., 2001; Young et al., 2012) (Figure 13).

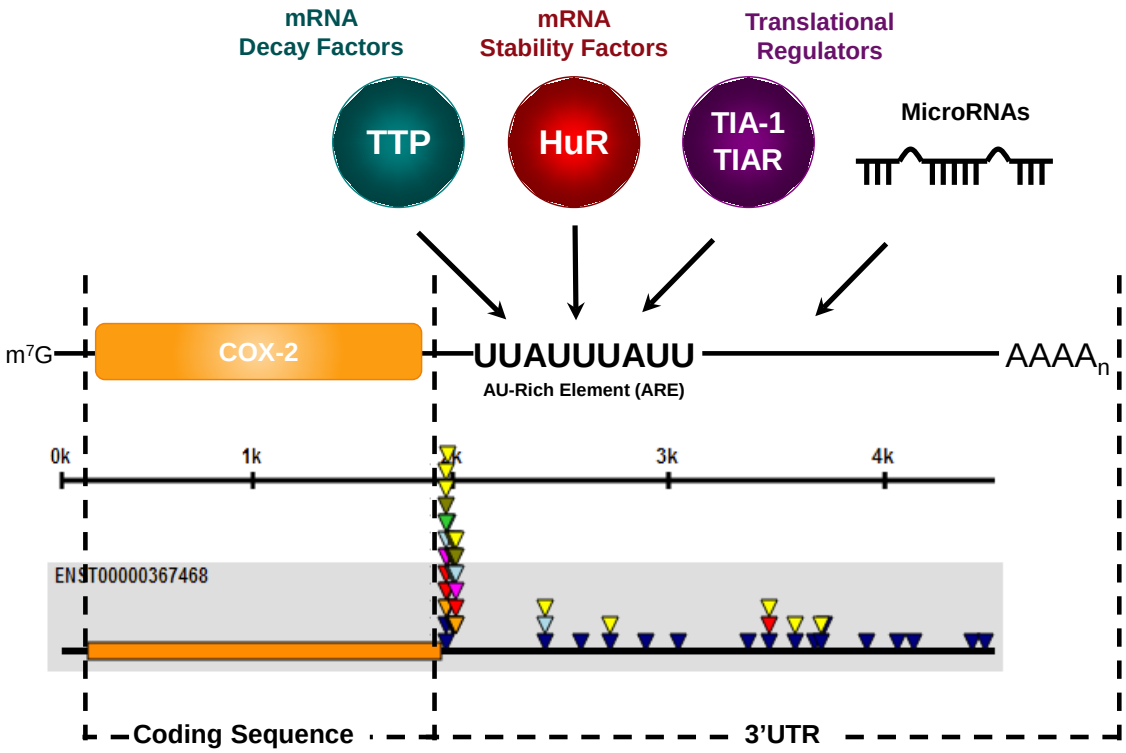

Figure 13. Schematic COX-2 transcript. *COX-2 mRNA with its coding sequence (in orange) and its 3'UTR (in black). In the COX-2 3'UTR, the AREs are shown (triangles in the low panel). Different post-transcriptional regulators of COX-2 expression are also shown (in the upper panel).*

HuR is primarily nuclear localized and contributes to the mRNA stabilization of many genes when exported to the cytoplasm (Figure 14). In the nucleus, HuR associates with the pre-mRNA introns and affects splicing and likely other nuclear processing events. HuR is exported to the cytoplasm with the mature mRNA where HuR stabilizes mRNAs, may help with transient mRNA storage (as in stress granules) and modulates the recruitment of the mRNA with the

translation machinery (polysomes) (Figure 14). Thus, HuR is implicated in a wide variety of physiological and pathological processes such as cell growth, differentiation and inflammation (Guhaniyogi and Brewer, 2001; Misquitta et al., 2001; Eberhardt et al., 2007; Meisner and Filipowicz, 2011).

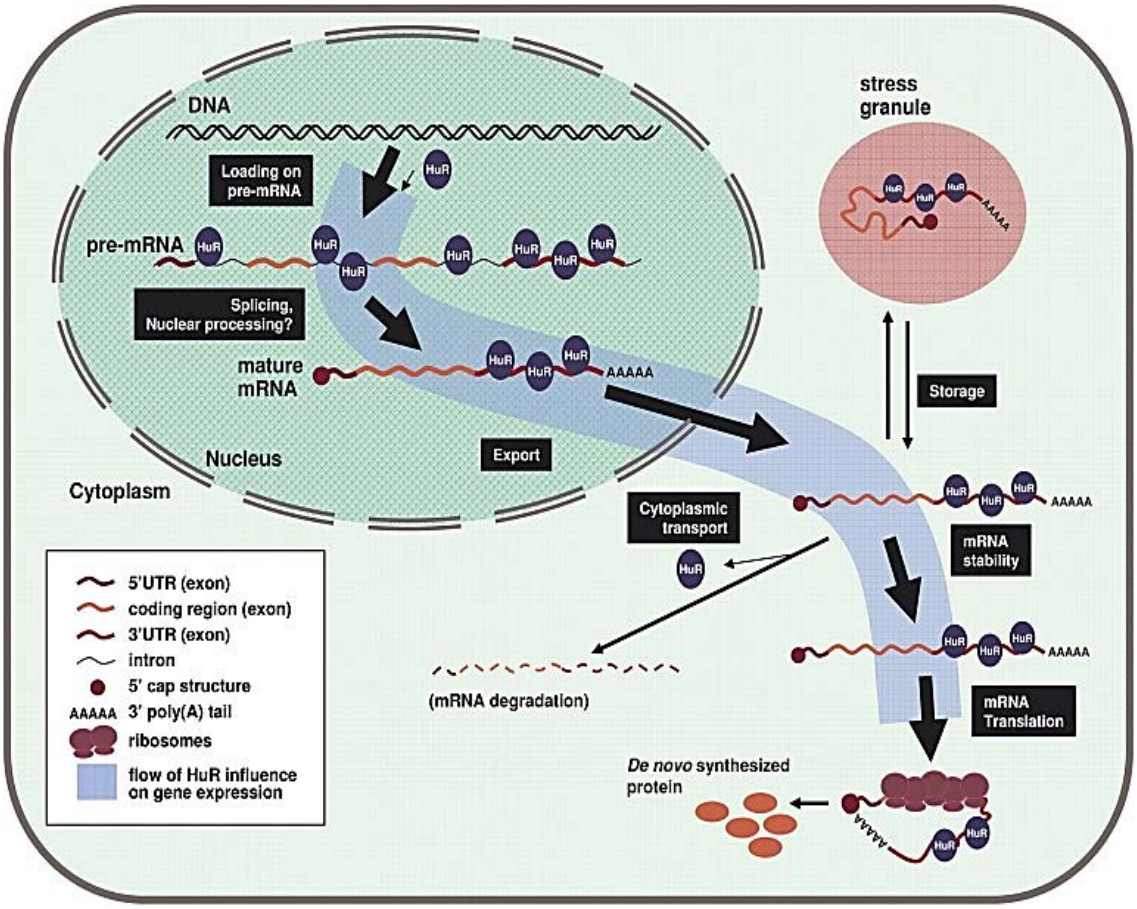

Figure 14. HuR shuttling and functions. *In the nucleus, HuR associates with the pre-mRNA introns (thin black lines) HuR is exported to the cytoplasm with the mature mRNA where HuR stabilizes mRNAs, may help with transient mRNA storage and modulates the recruitment of the mRNA with the translation machinery. Taken from Srikantan and Gorospe, 2011.*

HuR contains three RNA-recognition motif (RRM) domains. The third RRM domain (RRM3) is separated of the other two domains by a hinge region where the HuR-nucleo-cytoplasmic shuttling sequence (HNS) is located. RRM3 contributes to interactions with the poly-A tail of target mRNA and the two N-terminal tandem RRM domains can selectively bind AREs (Figure 15). Furthermore, RRM1 and RRM2 are involved in HuR dimerization, necessary for its binding to the AREs of mRNA targets. Some low-molecular-weight drugs, such as MS-444, bind to this region inhibiting the dimerization of the protein and

thus, HuR binding to the mRNAs (Meisner and Filipowicz, 2007; Meisner and Filipowicz, 2011).

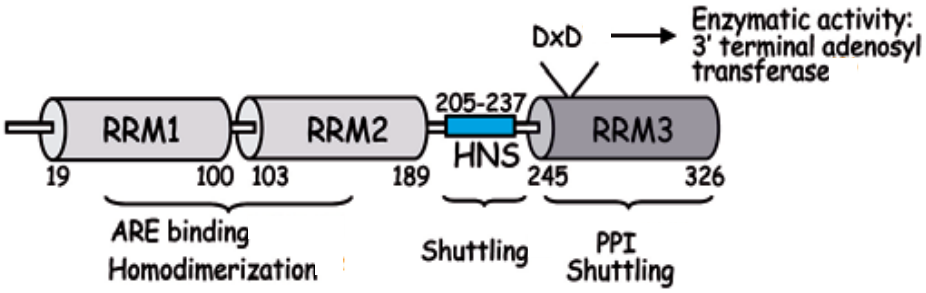

Figure 15. Schematic overview of HuR domain organization. *HuR domain organization and functional annotation. Modified from Meisner and Filipowicz, 2011.*

HuR can undergo different post-translational modifications which are involved in HuR subcellular location (Meisner and Filipowicz, 2011). These modifications include phosphorylation and methylation of serine and threonine residues and they are responsible for HuR nuclear-cytoplasmic shuttling (Meisner and Filipowicz, 2011) and activity (Yang et al., 2004b). Many proteins have been described to phosphorylate HuR such as checkpoint kinase 2 (Chk2) and protein kinase C (PKC) α or δ (Meisner and Filipowicz, 2011) (Figure 16). Moreover, in different pathologies including cancer, HuR cytoplasmic translocation is accompanied by an increase in HuR expression (Wang et al., 2013), thus HuR activity can be regulated by its expression or by post-translational modifications.

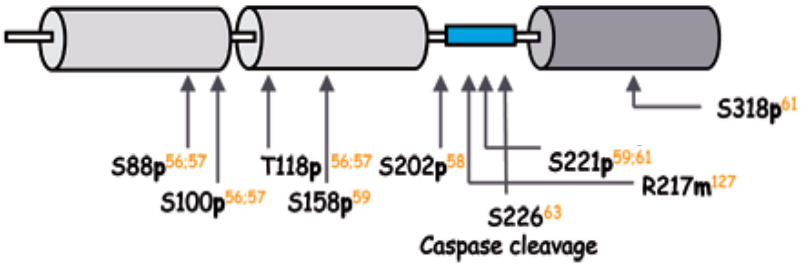

Figure 16. Positions of post-translational modifications of HuR*. Phosphorylation of S88, S100 and T118 is catalyzed by Chk2. S158 and S221 are phosphorylated by PKCα and S221 and S318 by PKCδ. S202 is a substrate for Cdk1. Arg217 is methylated by CARM-1. Finally, caspase 3 can cleave HuR at S226. Coactivator-associated arginine methyltransferase 1 (CARM-1). Modified from Meisner and Filipowicz, 2011.*

We have previously mentioned that HuR is involved in a number of physiological and pathological processes such as cell growth, differentiation and inflammation. However, data available in vascular cells are scarce. In human umbilical vein endothelial cells, HuR regulates stress-sensitive genes and HuR siRNA inhibits inflammatory responses including up-regulation of ICAM-1 and VCAM-1, NF-κB phosphorylation and adhesion of monocytes (Rhee et al., 2010). Moreover, in VSMC, HuR is translocated to the cytoplasm after PDGF stimulation and stabilizes different genes implicated in cell proliferation such as cyclin-dependent kinase 2 (Cdk2) (Pullmann et al., 2005). Although the ability of HuR to stabilize COX-2 mRNA in different cell types such as intestinal cancer cells, myeloid leukocytes or human mesangial cells has been described (Dixon et al., 2001; Dixon et al., 2006; Doller et al., 2008; Young et al., 2012), the ability of HuR to influence COX-2 expression in vascular cells is not yet explored. More importantly, whether HuR-dependent COX-2 stabilization has a role in VSMC migration is still unknown.

2.2.1.2. Prostanoids

As mentioned, prostanoids are metabolites of the arachidonic acid and play an important role in inflammation, platelet aggregation, vasoconstriction/relaxation and vascular remodeling. After the initial synthesis of the short-lived but biologically active PGH_2, the production of the different prostanoids (the prostaglandins PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2) depends on the activity of specific synthases; thus, prostanoid production depends on both COX and PGS activities (Figure 17). Prostanoids have different vascular effects depending on the activation of specific receptors. Prostanoids receptors are GPCRs and their nomenclature is based on the ligand bound by the receptor (Bos et al., 2004). Each prostaglandin receptor is coupled with a G_α subunit triggering G-protein-dependent cAMP and/or Ca^{2+} signaling that modulate a variety of transcription factors and intracellular signaling pathways involved in multiple cellular processes (Figure 17). We will now focus on the specific signaling pathways activated by each prostanoid receptor.

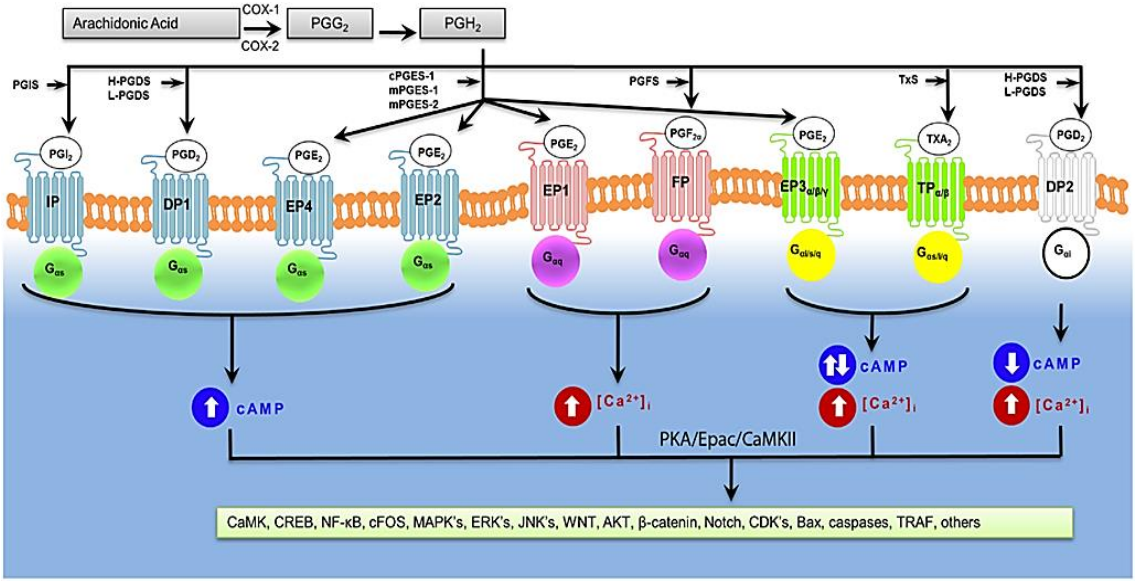

***Figure 17. Signal transduction mechanisms of prostaglandin receptors**. Prostaglandin receptors with their G_α-coupled subunit trigger G-protein-dependent cAMP and/or Ca^{2+} signaling. Taken from Mohan et al., 2012.*

Prostaglandin E_2 (PGE_2)

PGE_2 is a pro-inflammatory molecule produced by PGE_2 synthases (PGES) that has an important role in the control of vascular tone (Audoly et al., 2001) and structure (Wang et al., 2011b). There are three PGES isoenzymes, two microsomal isoforms (mPGES-1 and mPGES-2) and one cytosolic isoform (cPGES) all of them coded by different genes. In most tissues, cPGES is constitutively expressed and coupling between cPGES and COX-1 has been proposed suggesting that cPGES might be involved in cellular homeostasis (Helliwell et al., 2004). On the other hand, mPGES-1 is induced by inflammatory stimuli and is the principal source of PGE_2 (Cheng et al., 2006). Different stimuli are able to increase mPGES-1 expression and/or activity at vascular level. For example, in cultured VSMC and fibroblasts, LPS or IL-1β, but not AngII, induce mPGES-1 expression (Wang et al., 2006; Galán et al., 2011). On the other hand, Wang et al. (2008) showed that in the AngII-infused mouse model, mPGES-1 vascular expression is increased. Because of the synchronized induction in the presence of some pro-inflammatory stimuli, a coupling between COX-2 and mPGES-1 has been suggested (Murakami et al., 2000; Stichtenoth et al., 2001); however, this issue is still under debate (de Oliveira et al., 2008).

mPGES-1 promoter contains GC boxes which are binding sites for Sp1 and early growth response factor-1 (Egr-1) which are responsible for basal transcription of the mPGES-1 (Ekstrom et al., 2003). In addition, the promoter sequence was found to contain consensus binding sites for C/EBP*α* and -*β*, AP-1 and the CACCC-binding factor, as well as putative progesterone or glucocorticoid response elements. Moreover, NF-κB (Catley et al., 2003) and the interaction between Egr-1 and C/EBPβ, a novel distal enhancer element, are necessary for regulating IL-1β-induced mPGES-1 expression (Walters et al., 2012).

As mentioned, PGE_2 has diverse functions depending on the activation of specific receptors. PGE_2 receptors include EP_1, EP_2, EP_3, and EP_4, which are encoded by distinct genes. The four EP receptors bind PGE_2 with higher affinity than others prostanoids. Moreover, each EP receptor is coupled to different G-proteins (Bos et al., 2004) (Figure 18). The EP_1 receptor seems to activate G_q and leads to a PLC-dependent Ca^{2+} release and PKC activity, activating MAPKs. The EP_2 and EP_4 receptors activate G_s, which leads to the activation of cAMP/PKA/CREB pathway and at the same time impair MAPK activation. The EP_3 receptor activation leads to activation of $G_{i,}$ and then PLC/PKC and MAPKs due also to a decrease in cAMP levels (Bos et al., 2004) (Figure 18). It has also been suggested that the activation of EP_3 can activate G_s and G_q (Liebmann et al., 2000; Yamaoka et al., 2009).

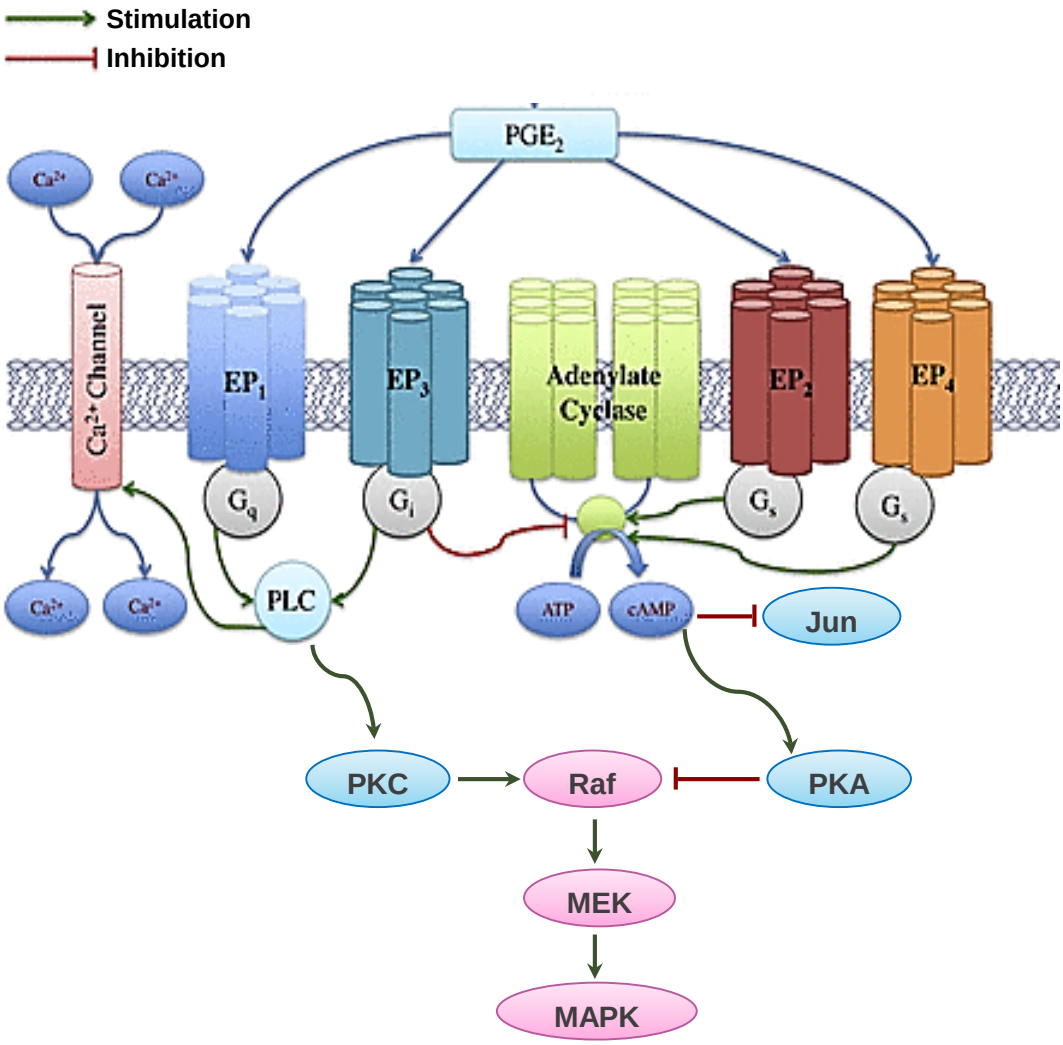

Figure 18. Activation of distinct EPs by PGE_2 induces several signaling pathways. *PGE_2 binds to distinct EPs to induce either activation of the MAPK pathway via G_q or G_i, or to induce up-regulation of cAMP and subsequently activation of PKA which leads to decreased MAPK activation. Mitogen-activated protein kinase kinase (MEK); Rapidly accelerated fibrosarcoma (Raf); Modified from Bos et al., 2004.*

EP_1 and EP_3 have been proposed to have an important role in vasoconstriction whereas EP_2 and EP_4 are important for vasorelaxation (Bos et al., 2004). Moreover, these receptors participate in proliferation and migration of vascular cells and depending on the receptor involved both inhibition and stimulation of cell proliferation or migration can be found (see below).

Thromboxane A_2 (TXA_2)

TXA_2 is a prothrombotic eicosanoid and a powerful vasoconstrictor and derived from PGH_2 through the activity of TXAS. TXAS promoter contains different binding sites for transcription factors such as AP-1, AP-2, GATA-1, CCAAT box, TATA box (Bos et al., 2004). The human TXA_2 receptor is the TP receptor and is coupled to the heterotrimeric G_q protein. TP activation leads to activation of MAPKs via G_q and PLC/PKC being essential for platelet aggregation, hypertrophy, VSMC contraction and proliferation (Ko, 1997; Bos et al., 2004). Alternatively, TP activation might lead to activation of MAPKs via Shc-GRB2-SOS, suggesting the participation of Ras in this activation (Bos et al., 2004). In parallel, TXA_2 induces PI3K/Akt, and thus, mTOR and p70 S6 kinase activation, which is involved in the inhibition of apoptosis (Morinelli et al., 1997) (Figure 19).

Vascular TXA_2 production is increased in several animal models of hypertension where it contributes to the increased contractile responses (García-Redondo et al., 2009; Martínez-Revelles et al., 2013). However, at vascular level, there is a paucity of information about which stimuli induce TXAS expression and TXA_2 production. Besides its effects on vascular contraction, TP activation seems to be linked to endothelial cell migration, survival and angiogenesis (Nie et al., 2000), although conflicting results have been found (Ashton et al., 1999). Importantly, in VSMC, TP activation mediates the proliferative effect of TXA_2 through phosphoinositide hydrolysis and ERK1/2 pathway, leading to DNA synthesis (Ko, 1997), which suggest that TXA_2 might have a role in vascular remodeling in cardiovascular pathologies.

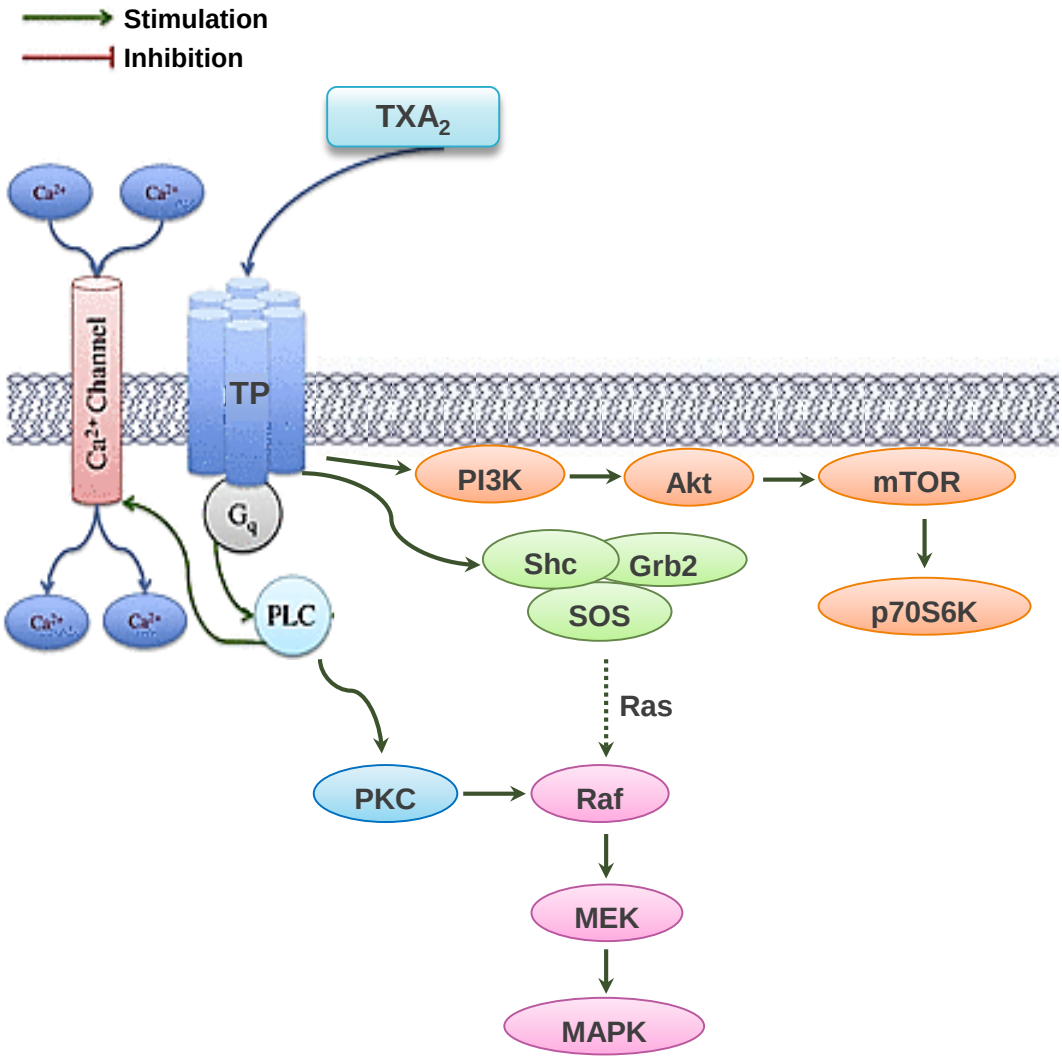

Figure 19. Signaling pathways involved in TP activation by TXA_2. *TXA_2 activates MAPK via G_q and PKC. Alternatively, it might lead to activation of this kinase via Shc-Grb2-SOS, suggesting the activation of Ras. In parallel, TXA_2 induces p70 S6 kinase activity. Mitogen-activated protein kinase kinase (MEK); Mammalian target of rapamycin (mTOR); Rapidly accelerated fibrosarcoma (Raf); Son of sevenless (SOS); Src homology 2 domain containing) transforming protein (Shc); Growth factor receptor-bound protein 2 (Grb2); Ribosomal protein S6 kinase (p70S6K). Modified from Bos et al., 2004.*

Prostacyclin (PGI_2)

PGI_2 is a vasodilator and antiplatelet aggregator prostanoid with vasoprotective functions that is generated by PGIS (Bos et al., 2004). PGIS is expressed in many human tissues including VSMC (Camacho et al., 2007). PGIS expression can be induced in vascular cells by different stimuli such as TNF-α (Murakami et al., 1993), hypoxia (Camacho et al., 2011), AngII (Galán et

al., 2011) or IL-1 (Fleisher-Berkovich and Danon, 1999); however, the regulatory elements of its promoter are poorly known. PGI_2 binds to IP receptor and stimulates G_s and activates cAMP/PKA/CREB resulting in elevated levels of cAMP which potently inhibit the MAPK pathways including ERK1/2 and p38 MAPK (Figure 20), as well as Rho kinase. In addition, IP activation can also activate G_q and G_i (Bos et al., 2004).

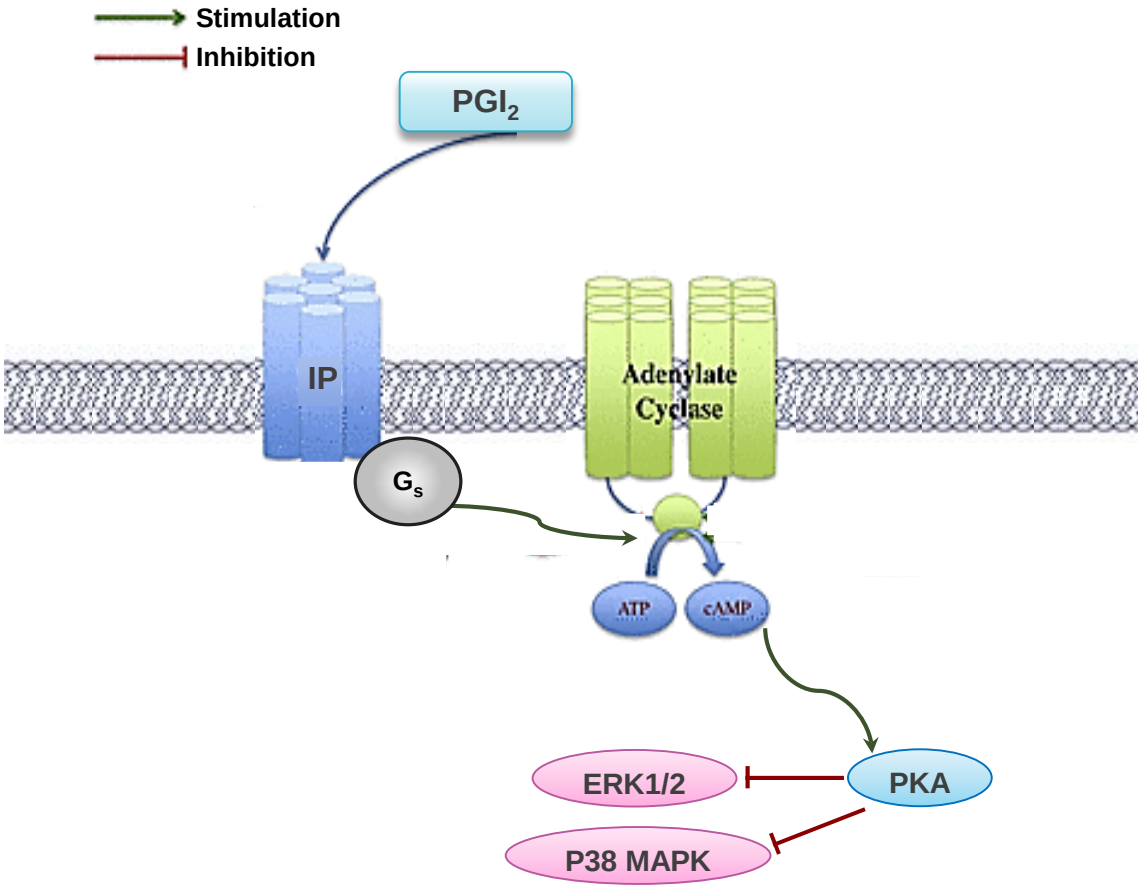

Figure 20. Signaling pathways involved in IP activation by PGI_2. *PGI_2 induces inhibition of both ERK1/2 and p38 MAPK via the activation of G_s. Modified from Bos et al., 2004.*

Besides inducing vasodilation, IP receptor activation inhibits VSMC proliferation and migration (Wang et al., 2011b). Thus, cicaprost, a selective IP agonist, inhibits cell cycle progression from G1-to S-phase of cultured mouse aortic VSMC by blocking the binding of CREB to the cyclin A promoter CRE and blocking cyclin E-cdk2 activity and phosphorylation of retinoblastoma protein and p107 (Kothapalli et al., 2003).

Role of prostanoids in vascular remodeling

In vivo studies using selective COX-2 inhibitors and COX-2$^{-/-}$ mice have demonstrated that prostanoids downstream of COX-2 participate in the pathological vascular remodeling observed in aortic aneurysms (King et al., 2006) and restenosis observed in the wire-injured (Zhang et al., 2013) and balloon-injured (Yang et al., 2004a; Wang et al., 2005) arteries. However, the implication of COX-2 in hypertensive vascular remodeling is less clear. Virdis et al., (2012) recently proposed that COX-1-derived prostanoids are responsible for AngII-mediated vascular damage of resistance arteries. However, we have demonstrated that COX-2 blockade prevents AngII-induced decreased aortic lumen size and increased media thickness (Martínez-Revelles et al., 2013). Of importance is the fact that cardiovascular side effects associated to COX-2-selective nonsteroidal anti-inflammatory drugs have been reported (reviewed in Patrignani and Patrono, 2014). However, there is still a big controversy of whether selective and non-selective COX-2 inhibitors provide the same degree of cardiovascular risk (Nissen, 2012; Bhala et al., 2013).

The main COX-derived prostanoids responsible for effects on vascular structure seem to be PGE_2 and TXA_2 with PGI_2 having a protective effect. Therefore, interest of the scientific community has shifted to the study of selective inhibitors of mPGES-1 as a drug target with previsible less cardiovascular side effects (Wang and Fitzgerald, 2010; Korotkova and Jakobsson, 2014). Unfortunately, to date there are no selective mPGES-1 inhibitors available for clinical use and some of the more selective and active for human mPGES-1 show no activity on rat or mice enzyme (Korotkova and Jakobsson, 2014). Preclinical studies using mPGES-1 knockout mice have provided promising results. Thus, global mPGES-1 deletion prevents abdominal aortic aneurisms development (Wang et al., 2008) and neointimal hyperplasia formation and impairs VSMC proliferation and migration after wire injury of the femoral artery (Wang et al., 2011b). However, recent studies have established that the effects of mPGES-1 on vascular damage might depend on the specific cell location of the enzyme being macrophage mPGES-1 a key enzyme in promoting neointimal hyperplastic response to injury (Chen et al., 2013a) and

atherogenesis (Chen et al., 2014). Interestingly, the role of mPGES-1-derived PGE_2 in hypertensive vascular remodeling is unknown.

Blockade of specific prostanoids receptors might provide more specific effects than the blockade of prostanoids synthesizing enzymes. However, data are still inconclusive and might depend on the stimulus or the specific cell type. Thus, in VSMC, PDGF-induced proliferation is mediated by EP_4 receptor (Fujino et al., 2002) and in injury-induced neointimal hyperplasia and PGE_2-induced VSMC migration, EP_3 seems to be a key player (Zhang et al., 2013). On the other hand, neointimal hyperplasia was markedly accelerated in $EP_2^{-/-}$ mice and PDGF treatment resulted in more significant cell proliferation and migration in VSMC of $EP_2^{-/-}$ mice (Zhu et al., 2011). In cardiac fibroblasts, PGE_2 stimulates proliferation via EP_1 and/or EP_3, ERK1/2 and Akt regulation of cyclin D3 (Harding et al., 2011). Regarding TP receptors, VSMC-specific TP deletion significantly attenuated AngII-induced hypertension and aortic vascular remodeling (Sparks et al., 2013). In addition, selective TP receptor blockade with the antagonist SQ29548 prevented small resistance arteries hypertrophy induced by AngII in mice (Virdis et al., 2012). Similarly, *in vitro* TP inhibition blocked vascular cell migration and proliferation induced by different stimuli (Sachinidis et al., 1995; Nie et al., 2000). Finally, as a proof of the protective role of PGI_2 in vascular structure is the fact that deletion of the IP receptor augments intimal hyperplasia in a mouse model of transplant arteriosclerosis or flow-induced vascular remodeling (Rudic et al., 2005).

The effects of prostanoids on vascular structure seem to be mediated by ECM proteins such as TN-C. Thus, the increased TN-C expression observed in the femoral artery wire injury mouse model is abolished in mPGES-1 knockout mice (Wang et al., 2011b) and PGI_2 reverses TN-C expression and vascular remodeling in rat pulmonary hypertension (Schermuly et al., 2005). Moreover, mPGES-1-deleted VSMC generated less PGE_2 but more PGI_2 and reduced TN-C expression, VSMC proliferation and migration *in vitro* compared with wild type cells (Wang et al., 2011b). However, whether TN-C is increased in hypertensive vascular remodeling and affected by PGE_2 is unknown.

2.2.2. NADPH oxidase

NADPH oxidases are the major source of ROS in the vascular wall in physiological and pathological conditions (Dikalova et al., 2005; Anrather et al., 2006; Lassègue et al., 2012; Montezano and Touyz, 2014). ROS are reactive derivatives of the oxygen metabolism with superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) being of major importance. They are produced by almost all cell types including vascular cells. The main catalytic function of NADPH oxidases is the generation of ROS, thus differing from the rest of the ROS-producing enzymes which produce ROS as a by-product of their activity. NADPH oxidase reduces oxygen to superoxide anion ($O_2^{\bullet-}$), being NADPH the electron donor; thus, there is an electron transfer from the cytosol across biological membranes. There are seven NADPH oxidases isoforms in mammals and all of them have a catalytic subunit called NOX (NOX-1-5) or DUOX (DUOX-1-2 also called NOX-6-7) and up to seven regulatory subunits (Figure 21).

NOX-1, NOX-2, NOX-4 and NOX-5 are expressed in the cardiovascular system. NOX-2 is the classical NOX that was primarily characterized in leukocytes. NOX-1, NOX-2 and NOX-3 activities are regulated by cytosolic adaptor proteins or “NOX organizers” (p47phox or NOXO1 and p40phox) and “NOX activators” (p67phox or NOXA1) that bind GTP-Rac and affect the flow of electrons (Figure 21). The p22phox component forms a stable heterodimeric complex with NOX core components (NOX-1-4), required for post-translation processing or maturation into active oxidases. In NOX-1/NOX-3 systems, p22phox also promotes plasma membrane targeting of the oxidases and provides a docking site for NOX organizers. However, NOX-4 only depends on p22phox in order to be active, is constitutively activated, and ROS production is regulated by Poldip2 (Figure 21). NOX-5 and DUOX are Ca^{2+}-responsive oxidases that contain Ca^{2+}-binding EF-hands (Figure 21). NOX-1, NOX-2, NOX-3 and NOX-5 produce $O_2^{\bullet-}$ while NOX-4, DUOX-1 and DUOX-2 produce H_2O_2 (Guichard et al. 2008; Montezano and Touyz, 2014).

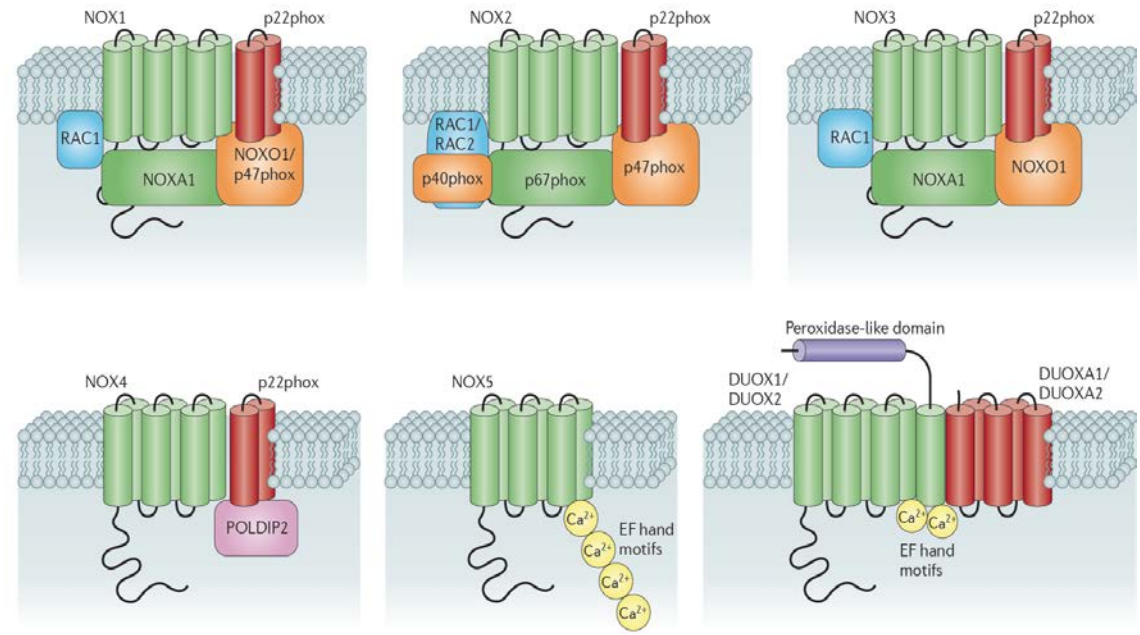

Figure 21. Subunit composition of the seven mammalian NADPH oxidase isoforms. *The catalytic subunits of NADPH oxidase (NOX) 1-5, dual oxidase (DUOX) 1 and 2 are shown in green. The stabilization subunits are shown in red: p22phox, DUOX activator (DUOXA) 1 and 2. Cytosolic organizers are shown in orange: p40phox, NOX organizer 1(NOXO1) and p47phox. Cytosolic activators are shown in green: p67phox and NOX activator 1 (NOXA1).Small GTPases (RAC1 and RAC2), polymerase δ-interacting protein 2 (POLDIP2) and EF hand motifs are shown in blue, pink or yellow respectively. Taken from Drummond et al., 2011.*

Within the vascular wall, NOX isoforms locations vary depending on the cell type and the cellular compartments (Drummond et al., 2011). Thus, endothelial cells express NOX-1, NOX-2, NOX-4 and NOX-5; VSMC mainly express NOX-1, NOX-4 and NOX-5; and adventitial fibroblasts mainly express NOX-2 and NOX-4 (Drummond et al., 2011). It is noteworthy that NOX-5 is only expressed in human cells (Drummond et al., 2011). NOX distribution in subcellular compartments also varies within the cell. In VSMC, NOX-1 is localized to the plasma membrane, caveolae and endosomes while NOX-4 seems to be in focal adhesions, ER and nucleus (Drummond et al., 2011; Chen et al., 2012) (Figure 22). Additionally, NOX-4 seems to be present in the mitochondria of cardiomyocytes (Chen et al., 2012).

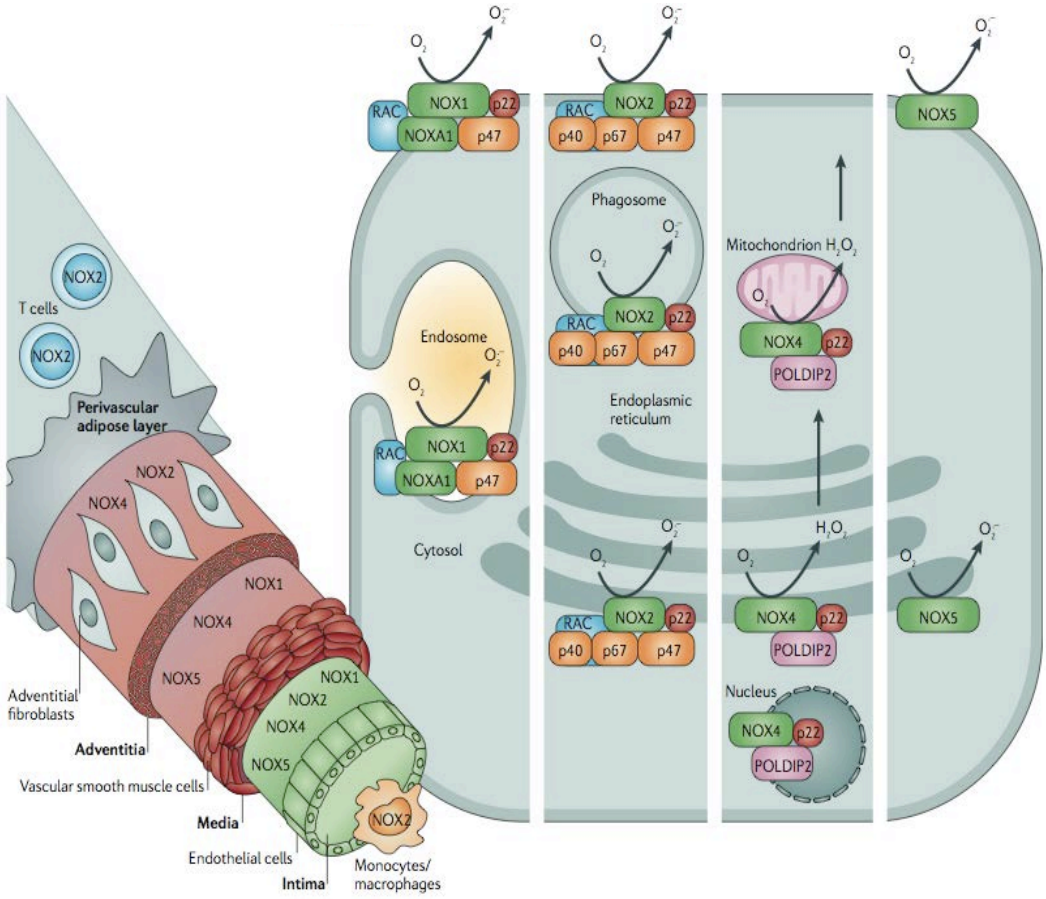

Figure 22. Cellular and subcellular expression of NADPH oxidase isoforms in the vessel wall. *Schematic diagram showing the subcellular location of the different NADPH oxidase isoforms (on the right) and their location in different cell types of the vascular wall (on the left). Taken from Drummond et al., 2011.*

NOXs are important in physiological processes including host defense, aging, and cellular homeostasis. However, the up-regulation of different NOXs, including NOX-1 and NOX-4, has been implicated in several cardiovascular diseases such as atherosclerosis, hypertension, diabetes, isquemia/reperfusion, restenosis or abdominal aortic aneurisms. Thus, NOX-derived ROS contribute to the oxidative stress, vascular inflammation, endothelial dysfunction and vascular remodeling observed in these cardiovascular pathologies (Dikalova et al., 2005; Anrather et al., 2006; Drummond et al., 2011; Lassègue et al., 2012; Montenzano and Touyz 2014; Raaz et al., 2014). The mechanisms whereby NOX-derived ROS contribute to altered vessel structure include modulation of cell growth, apoptosis, migration, inflammation and ECM production (Xu and Touyz, 2006; Drummond et al., 2011). This is based on both *in vitro* and *in vivo* studies using genetically modified animals and experimental models of

hypertension, atherosclerosis, aneurysms and others. However, although a causal relationship has clearly been demonstrated in many animal studies, an effective ROS-modulating therapy still remains to be established by clinical studies. In addition, despite of the amount of literature available on this subject, the regulation of specific NOXs in vascular cells is not completely understood.

Because of their preferential expression in VSMC and their importance in vascular remodeling, in the next part of the Introduction we will focus on specific aspects of NOX-1 and NOX-4 including available information on regulation, function and their role in vascular remodeling.

2.2.2.1. NOX-1

NOX-1 is expressed in colon epithelium and also in other tissues including the vascular wall where it seems to be up-regulated in pathological conditions or after exposure to different agonists important in cardiovascular disease (Lassègue et al., 2012). Thus, in VSMC NOX-1 is up-regulated by AngII (Lassègue et al., 2001; Briones et al., 2011), $PGF_{2\alpha}$ and PDGF (Katsuyama et al., 2005), IFN-γ (Manea et al., 2010a) or IL-1β (Martín et al., 2012). In addition, vascular NOX-1 expression is elevated in several *in vivo* animal models of hypertension such as two-kidney two-clip renovascular hypertensive rats, DOCA salt hypertensive rats and AngII-infused mice (Wang et al., 2007; Nakano et al., 2008; Martínez-Revelles et al., 2013). Moreover, NOX-1 expression is elevated during restenosis following balloon angioplasty (Szöcs et al., 2002). However, the role of NOX-1 in atherogenesis remains controversial with NOX-1 being undetected in atherosclerotic rabbit (Paravicini et al., 2002) or human lesions (Sorescu et al., 2002; Kalinina et al., 2002) and overexpressed in aorta from $ApoE^{-/-}$ mice (Bengtsson et al., 2003).

NOX-1 promoter has different binding sites for transcription factors including a member of CREB/ATF family (ATF-1; Katsuyama et al., 2005), AP-1 (Cevik et al., 2008), NF-κB (Manea et al., 2010b) or Janus kinase/Signal transducers and activators of transcription (JAK/STAT) (Manea et al., 2010a) (Figure 23).

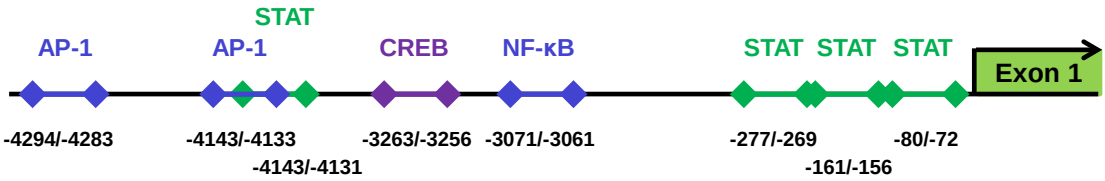

Figure 23. Structure of human NOX-1 gene promoter. *Binding sites for transcription factors involved in NOX-1 expression.*

Most of these studies have evaluated transcriptional regulation of NOX-1. However, to our knowledge, no studies have demonstrated post-transcriptional regulation of NOX-1. In fact, regulation of NOX-1 mRNA through its 3'UTR is conceivable because of the presence of AREs which, as mentioned, are implicated in mammalian mRNA degradation. Particularly, the role of HuR in stabilizing NOX-1 mRNA and whether this might affect NOX-1 activity and its functional properties has never been evaluated.

2.2.2.2. NOX-4

NOX-4 is very abundant in kidney and it seems ubiquitously expressed mainly in differentiated cells. NOX-4 is mostly found in focal adhesions and in the ER (Hilenski et al., 2004; Chen et al., 2008; Helmcke et al., 2009). As mentioned, its structure differs from NOX-1 and enables the protein to directly produce H_2O_2 (Dikalov et al., 2008; Takac et al., 2011). It has been suggested that the predominant factor controlling NOX-4-dependent ROS formation is the expression level of the enzyme (Schröder, 2014); therefore, the knowledge of the mechanisms responsible of its expression is very important.

It seems now accepted that NOX-4 is constitutively active (Chen et al., 2012). However, less clear is whether NOX-4 expression can be modulated and variable data regarding NOX-4 induction are found in the literature. Thus, hypoxia induces NOX-4 expression in pulmonary artery SMC (Mittal et al., 2007; Diebold et al., 2010) and TGF-β induces NOX-4 in cardiomyocytes and vascular cells (Cucoranu et al., 2005; Sturrock et al., 2006; Sturrock et al., 2007). However, thrombin, PDGF and peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands reduce NOX-4 expression in VSMC and endothelial cells, (Lassègue et al., 2001; Ellmark et al., 2005; Hwang et al.,

2005). Moreover, other stimuli including AngII and IL-1β have demonstrated to up-regulate, decrease or no affect NOX-4 expression in vascular cells (Lassègue et al., 2001; Ellmark et al., 2005; Dikalov et al., 2008; Richard et al., 2009; Briones et al., 2011). Reasons for these differences remain elusive but different locations in different cell types or presence of different NOX-4 isoforms might contribute to the observed findings (Lassègue et al., 2012).

In vivo studies have tried to shed light on the role of NOX-4 in cardiovascular disease; however, findings are still far from being conclusive. Depending on the pathology or the blood vessel studied, increased, decreased or unchanged NOX-4 expression can be found (Chen et al., 2012). Thus, in SHR, NOX-4 levels have been reported to be decreased in the aorta (Wind et al., 2010). In contrast, NOX-4 mRNA expression is higher in basilar arteries from SHR compared to normotensive Wistar-Kyoto rats (WKY) and in aortas from SHR (Paravicini et al., 2004; Martínez-Revelles et al., 2013). Similarly, increased NOX-4 expression has been observed in the renal cortex of aldosterone-salt rats and in aorta of AngII-infused mice (Mollnau et al., 2002; Nishiyama et al., 2004). In human atherosclerosis, NOX-4 expression is increased in intimal lesions of coronary arteries (Sorescu et al., 2002); however, in experimental atherosclerosis, NOX-4 expression is unchanged in the aorta of $ApoE^{-/-}$ mice or in primate models (Judkins et al., 2010; Stanic et al., 2012).

NOX-4 regulation seems to be mostly transcriptional (Figure 24). NOX-4 has been proposed to be a housekeeping gene because its promoter region contains many GC bases (Katsuyama et al., 2012). E2F1 transcription factor is involved in the basal NOX-4 expression in rodent VSMC (Zhang et al., 2008). Sp3 and three GC-boxes containing putative Sp/Klf binding sites are also essential for the basal expression of the NOX-4 gene (Katsuyama et al., 2011). Furthermore, in human endothelial cells, NOX-4 basal transcription is dependent of the deacetylation of transcription factor(s) and polymerase(s) (Siuda et al., 2012). Regarding the inducible expression of NOX-4, JAK/STAT and NF-κB seem to be involved in NOX-4 expression in response to IFN-γ or TNF-α (Manea et al., 2010a; Manea et al., 2010b). In addition, hypoxia induces NOX-4 through a hypoxia-inducible factor-1α (HIF-1α) dependent mechanism

contributing to maintain ROS levels in smooth muscle cells from pulmonary artery (Diebold et al., 2010). However, the mechanisms whereby NOX-4 is down-regulated are poorly understood. To date, it has been demonstrated that a PPAR-γ agonist inhibits hypoxia-mediated NOX-4 increase in smooth muscle cells from pulmonary artery (Lu et al., 2010). Additionally, JunD knockdown decreases NOX-4 expression in endothelial cells (Paneni et al., 2013). However, additional mechanisms might contribute to NOX-4 down-regulation in response to different stimuli.

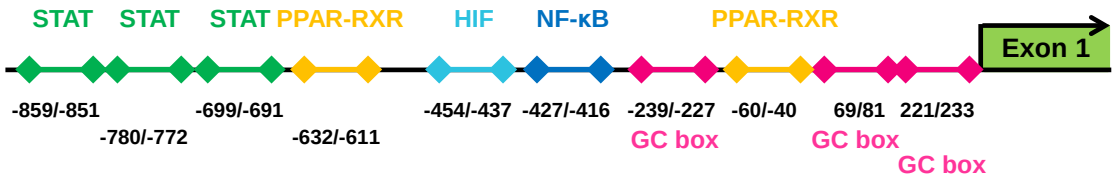

Figure 24. Structure of human NOX-4 gene promoter. *Binding sites for transcription factors involved in NOX-4 expression. Retinoid X receptor (RXR).*

In the last few years, NOX-4 post-transcriptional regulation has also been described. In cholesterol-fed rats, hypercholesterolemia down-regulates myocardial microRNA-25 expression and consequently increases NOX-4 expression and oxidative/nitrative stress in the heart (Varga et al., 2013). In addition, in cultured endothelial cells, serum stimulation decreases NOX-4 mRNA level due to a translation-initiated mRNA destabilization program (Peshavariya et al., 2009). Despite these evidences, there are very few studies focused on NOX-4 post-transcriptional regulation.

2.2.2.3. Reactive oxygen species

As mentioned, $O_2^{\bullet-}$, H_2O_2 and $ONOO^-$ are signaling molecules of major importance that are produced by almost all cell types including vascular cells. Besides NADPH oxidase, other sources of ROS in the vascular wall include mitochondria, xanthine oxidase (XO), uncoupled endothelial nitric oxide synthase (eNOS), ER, COX, cytochrome P450 and lipoxygenase (Frazziano et al., 2012; Hernanz et al., 2014). Mitochondria are a major cellular source of ROS. There are several sites in the electron-transport chain where oxygen can be reduced to $O_2^{\bullet-}$, with complexes I and III being the sites with the greatest

capacity (Dikalov, 2011). XO catalyzes the sequential oxidation of hypoxanthine to xanthine and xanthine to urate and can generate $O_2^{\bullet-}$ and H_2O_2 (Lacy et al., 1998). XO is mainly expressed in the endothelium and both its protein expression and $O_2^{\bullet-}$ production can be activated by AngII (Landmesser et al., 2007). eNOS uses L-arginine as substrate and tetrahydrobiopterin (BH_4) as cofactor to generate NO. However, under pathological conditions, L-arginine or BH_4 deficiency induce eNOS uncoupling resulting in $O_2^{\bullet-}$ production (Schulz et al., 2008).

$O_2^{\bullet-}$ is highly reactive, has a short half-life and is unable to diffuse across biological membranes except possibly via ion channels (Touyz and Briones, 2011). $O_2^{\bullet-}$ can dismute to H_2O_2, both spontaneously and enzymatically via any of the three isoforms of the superoxide dismutase (SOD): cytosolic Cu/Zn-SOD or SOD1, mitochondrial Mn-SOD or SOD2 and extracellular EC-SOD or SOD3 (Figure 25). As mentioned, H_2O_2 can also be formed directly by some types of NOX such as NOX-4, DUOX-1 and -2 (Drummond et al., 2011). H_2O_2 is more stable than $O_2^{\bullet-}$ and crosses membranes through some members of the aquaporin family (Al Ghouleh et al., 2013). H_2O_2 is rapidly metabolized to water and oxygen by several enzymatic systems such as glutathione peroxidase, catalase and the thioredoxin system (Ebrahimian and Touyz, 2008) (Figure 25). In the presence of transition metals (such as Fe^{2+}) H_2O_2 can be converted to hydroxyl radicals ($HO^{\bullet}$), which are highly reactive and can cause damage to lipids, proteins and DNA. In addition, NO which has a very short half-life, can react with $O_2^{\bullet-}$ to form $ONOO^-$ that is capable of modifying the structure and function of proteins. Thus, ROS regulation is important to maintain redox environment of the cell. When there is an imbalance between oxidants and antioxidant systems increased ROS steady-state levels start multiple pathologies including inflammation and cardiovascular disease (Drummond et al., 2011; Frazziano et al., 2012). At low intracellular concentrations, ROS have a key role in the physiological regulation of vascular tone, cell growth, adhesion, differentiation, senescence and apoptosis (Drummond et al., 2011; Schröder, 2014). However, an increase in the amount of ROS leads to pathological processes such as endothelial dysfunction, inflammation and proliferation or migration of VSMC leading to vascular remodeling.

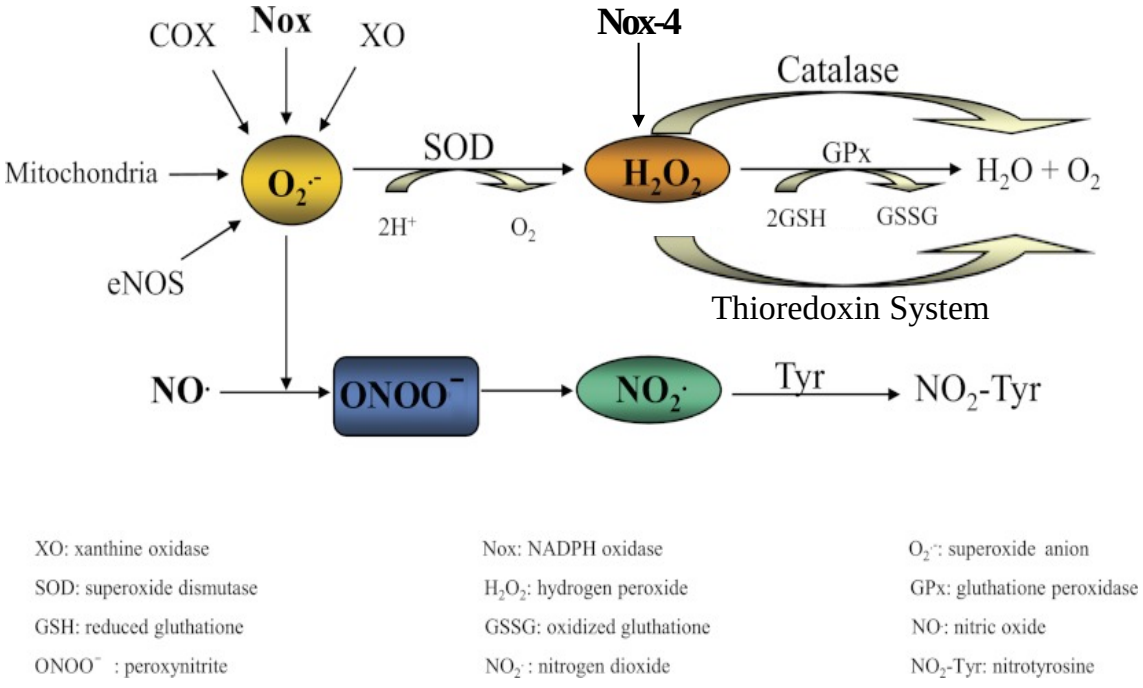

Figure 25. Schematic illustration of reactive oxygen and nitrogen species synthesis. *$O_2^{\cdot-}$ and H_2O_2 are synthesized by different enzymes and its reaction with NO generate the reactive nitrogen species $ONOO^-$ that could be converted into NO_2–, which reacts with protein tyrosine residues to generate NO_2-Tyr. Modified from Frazziano et al., 2012.*

ROS can act as second messengers activating different intracellular signaling pathways. Particularly, H_2O_2 induces post-translational oxidative modifications on sulfur containing amino acid of proteins such as methionine and cysteine residues. Thus, ROS react with the sulfur atom of cysteine side chains of different proteins including proteins implicated in cell migration. Depending on the environmental oxidative state these reactions are reversible or irreversible, causing protein oxidative damage. It is also well established, that redox-dependent signaling pathways in VSMC include modifications in the activity of protein tyrosine kinases such as Src, Ras, JAK2, Pyk2, PI3K and EGFR, as well as MAPK, particularly p38 MAPK, ERK1/2 and ERK5 which have a key role in cell migration and proliferation and hence in pathological vascular remodeling (Briones and Touyz, 2010, Figure 26). These processes probably occur through oxidation/reduction of protein tyrosine phosphatases (PTP), which are susceptible to oxidation and inactivation by ROS. Increased intracellular ROS also induces an increase in intracellular free Ca^{2+}

concentration ([Ca^{2+}]i) and an increase in intracellular pH (pH_i) that also contribute to altered contraction and remodeling observed in pathological situations where ROS have a prominent role (Briones and Touyz, 2010, Figure 26). Rho GTPases and actin are also sensitive to these modifications leading to actin cytoskeleton reorganization (Briones and Touyz, 2010; Son et al., 2011; Stanley et al., 2014). Thus, ROS are able to induce VSMC proliferation and migration by a number of different intracellular signaling pathways.

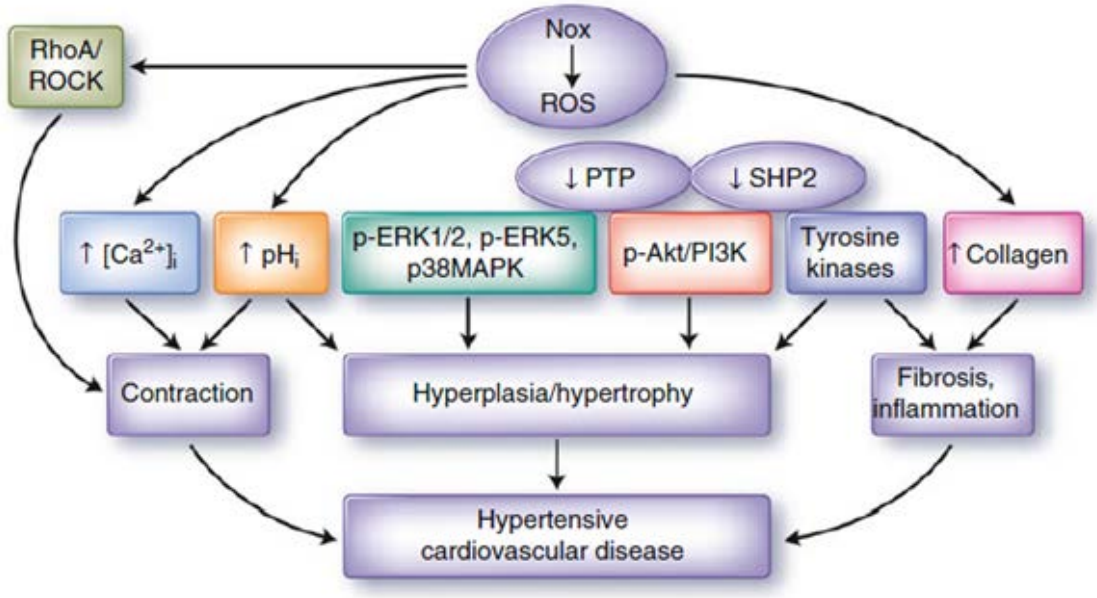

Figure 26. Intracellular mechanisms activated by ROS that participate in cardiovascular disease. *NOX-derived ROS activate different signaling pathways as well as increase in pH and Ca^{2+}. These processes lead to different cellular responses that will end in cardiovascular disease. Taken from Briones and Touyz, 2010.*

To date, a number of studies have demonstrated that important stimuli for cardiovascular diseases induce VSMC migration and/or proliferation *via* ROS (Amanso and Griendling, 2012). For example, AngII regulates FAT atypical cadherin 1 (Fat1) expression and activity and induces Fat1-dependent VSMC migration via activation of AT_1R, ERK1/2, and NOX-1-derived ROS (Bruder-Nascimento et al., 2014). Similarly, PDGF-induced VSMC migration is ROS dependent and the Src/PDK1/PAK1 signaling pathway is important as a ROS-sensitive mediator of migration (Weber et al., 2004). Moreover, in VSMC H_2O_2 induces cell migration by inducing the expression of a cytoskeleton protein, ARPC2, through a p38 MAPK-dependent mechanism (Al Ghouleh et al., 2013).

Role of reactive oxygen species in vascular remodeling

Many *in vitro* studies have demonstrated the role of oxidative stress as facilitator of different processes leading to vascular remodeling in cardiovascular diseases (Valente et al., 2012; Al Ghouleh et al., 2013; Bruder-Nascimento et al., 2014). In addition, several studies in different animal models including the SHR or the AngII-infused mice, have demonstrated that pharmacological treatment with different antioxidants such as tempol a SOD analogue, apocyinin, a non-specific NADPH oxidase inhibitor, or mito-TEMPO, a mitochondria targeted SOD mimetic, attenuated vascular remodeling (Park et al., 2002; Virdis et al., 2004; Chen et al., 2013b; Martínez-Revelles et al., 2013), demonstrating the key role of ROS from different origins in vascular disease.

Regarding the specific NOX isoform involved in vascular remodeling, transgenic knockout or overexpressing mice have yielded additional although not conclusive results. In the wire injury-induced neointima formation model, both proliferation and apoptosis were reduced in NOX-$1^{-/-}$ mice but there was little difference in mice overexpressing NOX-1 compared with wild type mice (Lee et al., 2009). Accordingly, proliferation and migration were reduced in response to PDGF in cultured NOX-$1^{-/-}$ VSMC and increased in cells overexpressing NOX-1 compared with wild type VSMC (Lee et al., 2009), suggesting that NOX-1 is required for the neointima formation. Several studies have evaluated the role of NOX-1 in vascular remodeling in response to AngII. AngII induces VSMC proliferation and migration as well as carotid artery hyperplasia in rats via AT_1R interaction with NOX-1 (Valente et al., 2012). Interestingly, NOX-$1^{-/-}$ mice showed a marked reduction in aortic media hypertrophy in response to AngII (Gavazzi et al., 2006; Schröder et al., 2012), but this reduction was due to a marked decrease in extracellular matrix accumulation and not in the number of VSMC (Gavazzi et al., 2006). On the other hand, Matsuno et al., (2005) demonstrated that AngII did elicit similar hypertrophic response in the thoracic aorta of NOX-$1^{-/-}$ and NOX-$1^{+/+}$ mice. Finally, transgenic mice overexpressing NOX-1 in SMC showed markedly greater aortic hypertrophy in response to AngII than their littermate controls (Dikalova et al., 2005). Altogether, these findings suggest that cell specific

location of NOX-1 might be the key to modulate hypertrophic vascular remodeling being VSMC NOX-1 of fundamental importance. Regarding mechanisms activated by NOX-1-derived ROS, it has been demonstrated that in the presence of some stimuli, NOX-1 activates different proteins involved in cell adhesion and migration such as paxilin, Rac, RhoA and cofilin (Schröder, 2014). Moreover, recently NOX-1 has been shown to be involved in matrix metalloproteinase-9 expression, a metalloproteinase essential in cell migration (Schröder, 2014).

The functional role of NOX-4 in vascular cells is under debate (Chen et al., 2012; Lassègue et al., 2012). It is thought that NOX-4 contributes to the maintenance of a differentiated state of the cell preventing cell activation or proliferation (Clempus et al., 2007; Schröder et al., 2009; Lassègue et al., 2012; Schröder, 2014), suggesting a protective effect of NOX-4. However, transgenic mice with cardiac specific overexpression of NOX-4 showed decreased left ventricular function with enhanced $O_2^{\bullet -}$, production in the heart, which was accompanied by increased apoptosis and fibrosis, suggesting a deleterious role for NOX-4 (Ago et al., 2010). Interestingly, NOX-$4^{-/-}$ mice developed exaggerated contractile dysfunction, hypertrophy and cardiac dilatation during exposure to chronic overload, whereas mice with cardiomyocyte-targeted overexpression of NOX-4 were protected (Zhang et al., 2010). The different functions of NOX-4 might also depend on the disease model or stimulus to be studied (Chen et al., 2012). In the AngII-infused mouse model, aortas from NOX-4-deficient animals developed increased inflammation, and media hypertrophy compared to their wild type littermates (Schröder et al., 2012) suggesting that NOX-4 might indeed act as a protective enzyme. Besides acting on differentiation, proliferation and migration, NOX-4 has a role on other processes involved in vascular remodeling such as apoptosis, senescence and cell cycle (Lassègue et al., 2012) and it has been suggested that NOX-4 might regulate fundamental cellular processes that contribute to each of these responses (Lassègue et al., 2012).

Reasons for so different roles for NOX-1 and NOX-4 in vascular biology are far for being clarified. As mentioned, NOX-4 is a special NOX because it has a

high constitutive activity, is highly expressed in some cells such as endothelial cells and its subcellular location is different to other NOXs (Chen et al., 2012). Moreover, different from NOX-1 and NOX-2, NOX-4 releases predominantly H_2O_2. Although not extensively studied, H_2O_2 in the media and endothelial layers may have different functions. Thus, smooth muscle-specific catalase overexpression blocks the H_2O_2-mediated angiotensin II-induced vascular hypertrophy (Zhang et al., 2005) whereas endothelial-specific catalase overexpression prevents exercise-stimulated induction of eNOS (Lauer et al., 2005). Future studies with improved tools will reveal the true nature of the role of NOX-4 in both health and disease (Chen et al., 2012).

2.2.3. Reciprocal relationship between reactive oxygen species and prostanoids

ROS and prostanoids share many stimuli and intracellular signaling pathways. Therefore, it is not surprising that both mediators could act in concert to induce their biological effects (Hernanz et al., 2014). Different studies have demonstrated that ROS from different sources can affect both the activity and expression of COX isoforms in vessels (Karaa et al., 2006; García-Redondo et al., 2009; Barbieri et al., 2011; Li et al., 2011; Martín et al., 2012; Martínez-Revelles et al., 2013). On the other hand, COX can produce directly ROS in part due to its ability to co-oxidize substances such as NADPH (Tang et al., 2007; Félétou et al., 2011). In addition, COX-derived products may function as autocrine stimulators of ROS (Hernanz et al., 2014). Selective COX-2 inhibitors and COX-2 deletion decrease vascular oxidative stress (Stichtenoth et al., 2005; Wu et al., 2005; Wu et al., 2011; Martínez-Revelles et al., 2013). However, it is unclear whether this effect is mediated by inhibition of COX-2-associated ROS production or by inhibition of the synthesis of downstream prostanoids that can directly regulate the expression and activity of several sources of ROS. Our group and others have reported a reciprocal relationship between COX-2 and NOX. Thus, in human liver cells phorbol 12-myristate 13-acetate initiates a pathway in which NOX-1 activation controls COX-2 expression and activity, which in turn induces NOX-4 expression by activation of EP_4 receptors (Sancho et al., 2011). At vascular level, antioxidants treatments

decreased COX-2 expression and activity in hypertensive animal models; conversely, selective COX-2 blockade decreased the increased oxidative stress associated to hypertension (Martínez-Revelles et al., 2013). More importantly, this reciprocal relationship had a role in the altered vascular function and hypertension (Martínez-Revelles et al., 2013). However, the impact of this relationship in vascular remodeling is unknown.

Aims

It is now accepted that inflammation is common for different cardiovascular diseases. This inflammatory process observed in both experimental animal models and in humans is associated with the increased expression and/or activation of different pro-inflammatory enzymes such as COX-2 and NADPH oxidase leading to the production of prostanoids and ROS. AngII and cytokines like IL-1β, promote a pro-inflammatory environment associated with increased COX-2 and NOX expression and/or activity in the vascular wall. As a result, VSMC undergo proliferation and migration contributing to vascular structural alterations observed in cardiovascular pathologies. Transcriptional and post-transcriptional mechanisms regulate COX-2 and NOX expression in response to inflammatory stimuli in different cell types. HuR, which is a RNA binding protein implicated in different cell processes, seems to have a key role in the stabilization of different mRNAs. However, whether HuR is involved in the stabilization of COX-2 and NOX mRNAs in VSMC is unknown. More importantly, whether this regulating mechanism has a functional role in VSMC is also unknown.

The aim of this PhD Thesis was to investigate transcriptional and post-transcriptional mechanisms implicated in the regulation of COX-2, NOX-1 and NOX-4 expression in VSMC stimulated with AngII and IL-1β. In addition, the role of this regulation in vascular remodeling was evaluated. The specific aims were as follows:

1. To analyze the contribution of HuR upon COX-2 expression induced by AngII and IL-1β and its consequences on VSMC migration and vascular remodeling.
2. To investigate transcriptional and post-transcriptional mechanisms involved in the effects of AngII and IL-1β on NOX-1 and NOX-4 expressions in VSMC and their implications in cell migration.

Materials and Methods

1. ETHICAL ASPECTS

All experimental procedures were approved by the Ethical Committees of Research of the Universidad Autónoma de Madrid and University of Kansas Medical Center and by the Reviewer Institutional Committee on Human Research of the Hospital de la Santa Creu i Sant Pau, conforms to the Declaration of Helsinki. These studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and with guidelines for ethical care of experimental animals of the European Community and the current Spanish and European laws (RD 223/88 MAPA and 609/86).

2. CELL CULTURE

Primary cultures of aortic VSMC were obtained from aortas of 3 months-old Sprague-Dawley male rats. Aortas were isolated and immediately submerged in DMEM-F12 medium supplemented with 0.1% BSA, 2 mmol/L L-glutamine and antibiotics (200 U/mL of penicillin and 200 µg/mL streptomycin). Aortas were cleaned in a cell hood (NuAIRE, Plymouth, MN, USA) and then treated with 2 mg/mL of collagenase type II (Worthington, Lakewood, USA) for 30 min at 37 ºC. After that, adventitia was removed with tweezers and the media layer was chopped and put into a well with DMEM-F12 medium supplemented with 10% FBS, containing 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Explants were incubated in a 6-well plate with complete medium at 37 ºC, 5% CO_2 and 95% air. Three times a week, the medium was replaced with fresh medium. 10-13 days later, confluent cells were rinsed with phosphate-buffered saline (PBS), split with trypsin-EDTA and seeded at a density of 30% in DMEM-F12 medium. Cells were identified as smooth cells by their spindle shape, by the “hills and valleys” organization and by immunocytochemical staining with specific monoclonal anti-α actin antibody (1:200; Sigma-Aldrich, St. Louis, MO, USA) (Figure 27). Rat cell cultures were used between passages 2 and 4.

A **B**

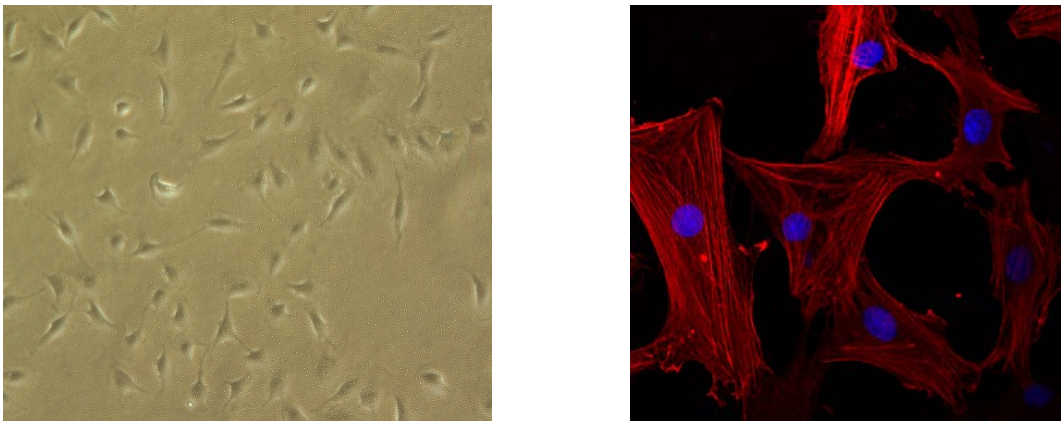

Figure 27. Vascular smooth muscle cells characterization. *Phase contrast* ***(A)*** *and α-actin staining (in red) (****B****) pictures of VSMC. Pictures were taken with a microscope Nikon with 10X objective or confocal microscope Leica TCS SP2 with 63X objective.*

Human VSMC were obtained from coronary arteries of hearts (removed in transplant operations) by using a modification of the explant technique (Orriols et al., 2014). Human VSMC were growth in M199 supplemented with 20% FBS, 1% human serum, 2 mmol/L of L-glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin. Human cell cultures were used between passages 3 and 6.

Cells were starved in serum free media for 24 h prior to stimulation. All cell culture products were purchased to Sigma-Aldrich (St. Louis, MO, USA).

Cells were stimulated with vehicle, AngII (0.1 µmol/L), IL-1β (10 ng/mL), AngII+IL-1β, 16,16-dimethyl PGE_2 (0.1-10 µmol/L) or U46619 (0.01-1 µmol/L) at the times and concentrations indicated in the results section. The effects of the following inhibitors were analyzed by its addition 30 min (or 4 h in the case of MS-444) before and throughout stimulation: actinomycin D, celecoxib, chelerythrine, cycloheximide, furegrelate, KN93, L798106, LY294002, ML171, MS-444, ozagrel, PD98059, suberoylanilide hydroxamic acid (SAHA), SB203580, SC19220, SP600125, SQ29548, trichostatin, tyrphostin AG 1478, U0126, W7, W12 and *5*-aza-2'-deoxycytidine (*5*-aza-*dC*) (Table 1). Compounds used for stimuli and ozagrel were dissolved in distilled water; remaining compounds were dissolved in DMSO. All compounds were prepared in a stock solution and in the day of the experiment they were diluted using DMEM-F12 medium.

INHIBITOR	CONCENTRATION	TARGET
Actinomycin D	5 µg/mL	DNA*[a]
Celecoxib	10 µmol/L	COX-2
Chelerythrine	20 µmol/L	PKC
Cycloheximide	25 µg/mL	*[b]
Furegrelate	10 µmol/L	TXAS
KN93	20 µmol/L	Ca2+/calmodulin-dependent protein kinase II (pCaMKII)
L798106	1 µmol/L	EP_3 receptor
LY294002	10 µmol/L	PI3K
ML171	0.5 µmol/L	NOX-1
MS-444	8 µmol/L	HuR
Ozagrel	10 µmol/L	TXAS
PD98059	20 µmol/L	MEK*[c]
SAHA	5 µmol/L	Histone deacetylase (HDAC) class I and II
SB203580	10 µmol/L	p38 MAPK
SC19220	10 µmol/L	EP_1 receptor
SP600125	20 µmol/L	JNK
SQ29548	3 µmol/L	TP receptor
Trichostatin	0.5 µmol/L	HDAC class I and II
Tyrphostin AG 1478	10 µmol/L	EGFR
U0126	10 µmol/L	MEK*[c]
W7	10 µmol/L	Calmodulin (CaM)
W12	10 µmol/L	CaM
5-aza-dC	2 µmol/L	DNA methyltransferase

Table 1. Inhibitors, final concentration and target.

**[a]*. Actinomycin D inhibits transcription through its binding to DNA at the transcription initiation complex and preventing elongation of RNA chain by RNA polymerase.*

**[b]*. Cycloheximide exerts its effect by interfering with the translocation step in protein synthesis (movement of two tRNA molecules and mRNA in relation to the ribosome) thus blocking translational elongation.*

**[c]*. PD98059 and U0126 block selectively the activation of MEK, thereby inhibiting the phosphorylation and the activation of ERK1/2.*

3. ANIMAL MODELS

The following animal models were used:

1) Model of left carotid artery ligation. C57BL6 male 4-month-old mice ~~with~~ were used. Mice were not allowed to eat 4h before the surgery. An anesthetic cocktail (ketamine 45 mg/kg plus medetomidine 0.6 mg/kg) and analgesic (buprenorphine 0.1 mg/kg) were administered intraperitoneally. The decrease of the corporal temperature was prevented with an electric blanket and eyes were protected from desiccation with an ophthalmic solution. The ventral neck region was shaved and disinfected with povidone-iododine solution. Once the animals were sedated to a surgical level of anesthesia (verified by toe or tail pinch), 1-cm incision was made in the neck (0.5 cm below the neck) and skin, glands and underlying muscular layer were separated with a retractor. Then, the left carotid was dissected and ligated with a 7-0 silk suture just proximal to its bifurcation of the left common carotid artery (Figure 28A). The ligation was performed in the left carotid in order to obtain an injured area of 9-mm length subjected to hemodynamic stress since the blood cannot escape. The right carotid was not used because blood could escape through the right subclavian artery thus the injury size would be smaller (Figure 28B). Mice were sutured with a 5-0 silk and atipamezol was administered intraperitoneally to recovery the animals of the anesthesia. During two days post-surgery buprenorphine (1.5 mg/L) was added in the drinking water.

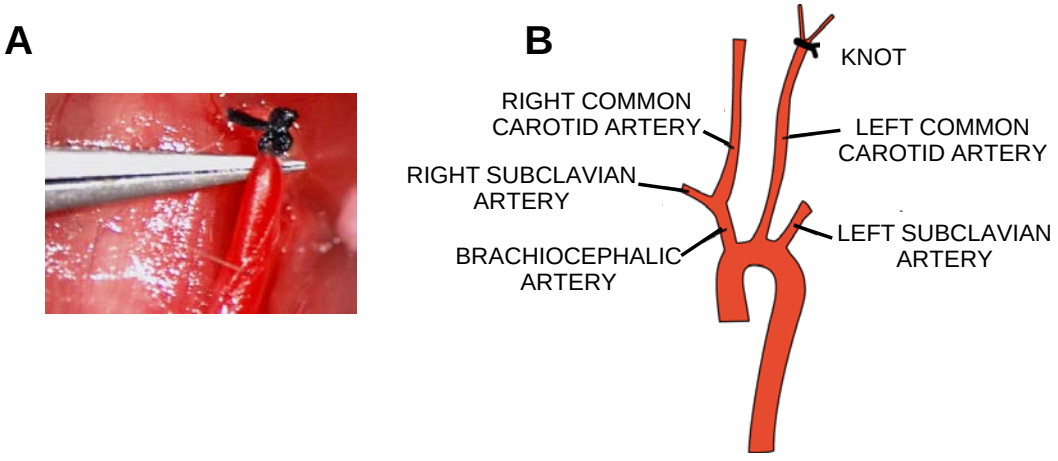

Figure 28. Left common carotid artery ligation surgery. A, *Left common carotid artery ligated.* ***B,*** *Scheme of the aortic arch, carotid arteries and the ligation site.*

2) Model of AngII infusion. C57BL6 male 4-month-old mice were infused with saline, AngII (1.44 mg/Kg/day, 2 weeks, subcutaneously by osmotic minipumps, Alza Corp., Cupertino, CA, USA) or with AngII plus celecoxib (25 mg/Kg/day intraperitoneal started 24 h before AngII-infusion). Mice were sedated with inhalational anesthesia (breathing concentrations of 2.5% isoflurane with oxygen) to a surgical level of anesthesia, verified by toe or tail pinch. After that, mid-scapular incision was made (Figure 29A), hemostat was inserted into the incision and, by opening and closing the jaws of the hemostat, the subcutaneous tissue was spread to create a pocket for the pump. The AngII filled pump was inserted into the pocket (Figure 29B). The wound was closed with 2-0 silk suture.

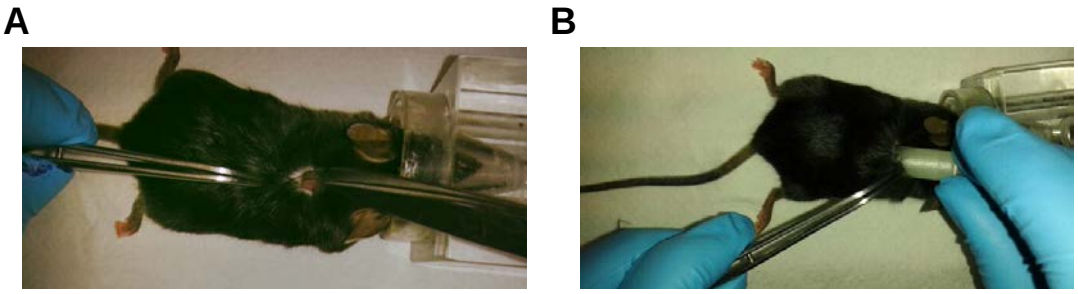

Figure 29. Osmotic mini-pump subcutaneous implantation. *Mid-scapular incision* ***(A)*** *and pump implantation* ***(B)*** *procedures.*

mPGES-1 wild-type and mPGES-1-deficient (mPGES-$1^{-/-}$) mice infused with saline or with AngII were also used. Wild type and mPGES-$1^{-/-}$ mice were maintained in a DBA/1 genetic background and were generated by corresponding heterozygous breeders derived from mPGES-$1^{+/-}$ embryo, which were a kind gift from Pfizer Inc (Groton, CT, USA) (Figure 30).

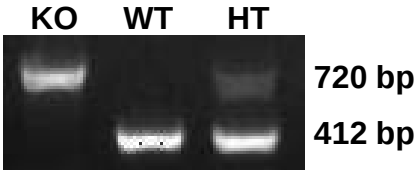

Figure 30. Genotyping of mPGES-$1^{-/-}$ (KO), wild type (WT) and heterozygous (HT) by PCR analysis. *The lower band (412 bp) is amplified from the wild type alleles and the upper band (720 bp) is from the mPGES-$1^{-/-}$ alleles.*

4. RNA ANALYSIS

For VSMC mRNA extraction, cells were rinsed with cold PBS and then harvested in 1 mL of Tri Reagent (Sigma-Aldrich) and transferred to a 1.5 mL tube. For aorta mRNA extraction, mouse aortas were homogenized on ice in 1 mL of Tri Reagent using a Polytron PT-20 (Kinematica AG, Lucerna, Switzerland). Samples were centrifuged 5 min 12,000 g to remove the debris of tissue, supernatants were transferred to a fresh tube and total RNA was obtained according to the manufacturer's recommendations. Total RNA absorbance was determined using a spectrophotometer Nanodrop (Thermo Fisher Scientific Inc, Wilmington, DE, USA) at 260 nm (1 unit A260 de ssRNA = 40 µg/mL). Sample absorbances at 280 and 230 nm were also determined in order to check RNA quality. 1 µg of total RNA was reverse transcribed using High Capacity cDNA Archive Kit (Life Technologies Inc., Gaithersburg, MD, USA) with random hexamers in a final volume of 10 µL according to manufacturer's recommendations. The reverse transcription PCR protocol was 10 min at 25 ºC, 2 h at 37 ºC and 5 min at 85 ºC.

Quantitative PCR (qPCR) was performed in a 7500 Fast ABI System (Life Technologies). Depending on the gene, primers for SyBR Green or TaqMan Probes were used. The final volume of the reaction was 10 µL composed by 5 µL of sample (50 ng), 4 µL of iTaq FAST SyBRGreen Supermix or 4.5 µL of iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA, USA) and 1 µL of forward and reverse primers or 0.5 µL of each probe. SyBRGreen primers concentrations were tested with a standard curve to obtain efficiency between 90-110%. We used Taqman Gene Expression Assays for human COX-2 (Hs00153133_m1), mPGES-1 (Hs00610420_m1), NOX-1 (Hs00246589_m1), NOX-4 (Hs00418356_m1), PGIS (Hs00168766_m1), TXAS (Hs01022706_m1), 18S (Hs99999901_s1), rat PPAR-γ (Rn00440945_m1), TXAS (Rn00562160_m1) and mouse TXAS (Mm00495553_m1) (Life Technologies). SYBR green primers sequence information is listed on Table 2.

Gene	Specie	FW	RV	Concentration (nmol/L)
COX-2	rat	AAGGGAGTCTGGAACATTGTGAAC	CAAATGTGATCTGGACGTCAACA	1.300
Egr-1	rat	CAGCGCTTTCAATCCTCAAG	GCGATGTCAGAAAAGGACTCTGT	500
Elf-3	rat	CTGAGCAAAGAATACTGGGACTGTC	CCATAGTTGGGCCACAGCCTCTGAAC	500
Elk-1	rat	GCTCCCCACACATACCTTGA	CGCGGTGCAATTGGACTCAGA	500
HuR	rat	TGTACATCAGTGGGCTTCCA	CTGCTTCAGACCGTTTGTCA	500
mPGES-1	rat	AGGAGTGACCCAGATGTG	ATGTATCCAGGCGATGAGA	340
NOX-1	rat	CGGCAGAAGGTCGTGATTA	TGGAGCAGAGGTCAGAGT	500
NOX-4	rat	GCCTCCATCAAGCCAAGA	CCAGTCATCCAGTAGAGTGTT	500
PGIS	rat	CCATCAACAGCATCAAACAGTTT	CAAAGCCATATCTGCTAAGGTCAA	500
TN-C	rat	ACCTCTCTGGAATTGCTCCCA	CATCTGAAACTAGAAGGTTGTC	500
B2M	rat	ACCGTGATCTTTCTGGTGCTT	TAGCAGTTGAGGAAGTTGGGC	110
COX-2	mouse	TTCGGGAGCACAACAGAGT	TAACCGCTCAGGTGTTGCAC	500
HuR	mouse	ATGAAGACCACATGGCGGAAGAC	AGTTCACAAAACCGTAGCCCAAGC	700
mPGES-1	mouse	AGGATGCGCTGAAACGTGGAG	CCGAGGAAGAGGAAAGGATAG	800
B2M	mouse	ACCCTGGTCTTTCTGGTGCTT	TAGCAGTTCAGTATGTTCGGCTT	200

Table 2. *SyBR Green primer sequences with the name of the target gene, the specie and the final concentration. β2-microglobulin (B2M).*

PCR cycles proceeded as follows: initial denaturation for 30 s at 95°C followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Melting curve analysis was performed in SYBR green reactions to show PCR product specificity. Relative expression was determined using $2^{-\Delta\Delta Ct}$ method (where C_t is threshold cycle) (Livak y Schmittgen, 2001), using β_2-microglobulin or 18S rRNA as the internal control. Variations of mRNA levels were calculated as fold increase over controls or over controls with the corresponding inhibitor.

Determination of COX-2, NOX-1 and NOX-4 mRNA stability in stimulated cells was initiated by adding 5 µg/mL actinomycin D, an inhibitor of transcription, to the growth medium at specified times. Then, total RNA was isolated at indicated times and COX-2, NOX-1, NOX-4 and β_2-microglobulin mRNA expression levels were measured by qPCR as indicated above.

5. WESTERN BLOT ANALYSIS

After appropriate treatments, cells were washed once in ice-cold PBS, scraped and whole-cell lysates were prepared in RIPA buffer containing 50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1% Nonidet-P40 (NP-40), 0.5% sodium deoxicolate and 1% sodium dodecyl sulfate (SDS). RIPA buffer was supplemented with a protease inhibitor cocktail (Roche Applied Science, Barcelona, Spain) and a mix of phosphatase inhibitors (1 mmol/L orthovanadate, 20 mmol/L β-glycerophosphate, 10 mmol/L NaF). Cell lysates were centrifuged 10 min 14.000 rpm and supernatants were transferred to a fresh tube.

For cellular fractionation, cells were lysed in hypotonic lysis buffer (10 mmol/L HEPES pH 8, 3 mmol/L $MgCl_2$, 40 mmol/L KCl, 0.2% NP-40, 10% glycerol, 0.1 mmol/L dithiothreitol (DTT) with protease and phosphatase inhibitors) for 15 min at 4 ºC. Samples were centrifuged 2 min at 10,000 rpm to separate cytoplasmic supernatant from nuclei. Nuclei were washed four times in hypotonic buffer and lysed in RIPA buffer. Samples were then centrifuged 10 min at 10,000 rpm to obtain the nuclear fraction.

Protein concentration was determined with Lowry (Bio-Rad) or BCA protein assay reagent (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as standard. 20-40 µg of protein were mixed with Laemli 5X buffer (300 mmol/L Tris-HCl pH 6.8, 10% SDS, 25% β-mercaptoethanol, 0.5% bromophenol blue, 50% glycerol) and boiled 5 min. Proteins were separated in a 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) submerged in running buffer (25 mmol/L Tris, 200 mmol/L glycine, 1% SDS). Then, gels were electrophoretically transferred to polyvinylidene difluoride or nitrocellulose membranes (Amersham, GE Healthcare, Buckinghamshire, UK) in Tris-Glycine transfer buffer (25 mmol/L Tris, 190 mmol/L glycine, 0.05% SDS,

20% methanol) in a Trans-Blot Cell (Bio-Rad). Membranes were blocked with 5% skim milk or 5% BSA in wash buffer (TBS-T: 10 mmol/L Tris-HCl pH 7.5, 100 mmol/L NaCl, 0.1% Tween-20) 30 min at room temperature. After that, membranes were probed with antibodies against HuR (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), NOX-1 and NOX-4 (1:200; Santa Cruz Biotechnology, Inc.), Nucleoporin p62 (1:5,000; BD Bioscience, San Jose, CA, USA), COX-2 and mPGES-1 (1:200; Cayman Chemical, Ann Arbor, MI, USA), PGIS (1:1,000; Cayman Chemical), p-myosin light chain (MLC) and MLC, p-ERK1/2 and ERK1/2, p-p38 MAPK and p38 MAPK, p-JNK and JNK, p-Akt and Akt (1:1,000; Cell Signaling, Boston, MA, USA). Detection was accomplished using horseradish peroxidase-coupled anti-rabbit (1:2,000; Bio-Rad) or anti-mouse (1:5,000; Stressgen, Victoria, Canada) IgG antibodies for 1 h at room temperature. Blots were stripped and then probed with antibodies against β-actin (1:50,000; Sigma-Aldrich) or tubulin (1:10,000; Sigma-Aldrich). Signal was detected using the Luminata Forte (Millipore Corporation, Billerica, MA, USA) detection system. Immunoblot signals were quantified using NIH ImageJ using β-actin or tubulin expressions as loading controls. Nucleoporin and tubulin were used to discard nuclear or cytoplasmic contamination in cytoplasmic or nuclear fractions, respectively. Variations of protein expressions were calculated as fold increase over controls.

6. RIBONUCLEOPROTEIN COMPLEX IMMUNOPRECIPITATION

Ribonucleoprotein complex immunoprecipitation was performed as described (Lal et al., 2004). Cells were lysed in polysome lysis buffer (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L DTT, 0.5% NP-40, 1 mmol /L PMSF, protease inhibitors, 100 U RNAse inhibitor). 200 µg of cytoplasmic lysate were incubated with anti-HuR antibody or control IgG precoated to protein A/G PLUS agarose (Santa Cruz Biotechnology) overnight at 4°C in NT2 buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 0.05% NP-40) adding 0.1 mmol/L DTT, 40 U RNAse inhibitor, 5 mmol/L EDTA and 100 µg/mL tRNA. Immunoprecipitates were collected by centrifugation and washed 4 times with NT2 buffer. Total RNA was isolated from immunoprecipitates using 1 mL TRIzol reagent and then used for cDNA synthesis and qPCR as described above.

7. PLASMID CONSTRUCTS

Constructs included in Figure 31 were performed in our laboratory or kindly provided by other groups.

The 537 bp of NOX-4 3'UTR DNA fragment was obtained by a nested PCR using human VSMC gDNA as a template and the following sets of primers, first primer set: FW: CATACTTCTTGTCATGTTCTGC, RV: TTTGAGGAAGGTTCAGTTAGC and XbaI-containing second primer set: FW: GCTCTAGAAAACTGAGTAACCAGAACAAC, RV: GCTCTAGATTTGCCTGGAGTGCTTGC. 1% agarose gel was used to separate the second PCR product and gel-purified (Qiagen, Valencia, CA, USA). The product was ligated overnight at 16 ºC to 100 ng of pGEM-T (Promega Corporation, Madison, WI, USA) in a ratio 1:3 (vector:insert) with T4 DNA ligase (New England Biolabs, Beverly, MA, *USA*), transformed in competent XL1-Blue bacteria (Stratagene, La Jolla, CA, USA) and plated on Petri dishes containing agar. Clones were selected 16 h with ampicillin as well as X-Gal and IPTG at 37ºC (blue-white screening). White colonies were picked up using a toothpick and allowed to expand (20 h, 37ºC, 250 rpm) in LB medium with 100 µg/mL of ampicillin. Plasmid DNA were isolated from each colony using a miniprep kit (Qiagen) and submitted to automated sequencing. 1.3 µg of positive colony DNA were digested with XbaI restriction enzyme (Roche Applied Science) at 37 ºC for 2 h. and the DNA fragment gel-purified. pGL3 control vector (Promega Corporation, Madison, WI, USA) was digested by XbaI, dephosphorylated with alkaline phosphatase (SEAP, Roche Applied Science), gel-purified and 100 ng were ligated in a ratio 1:3 (vector:insert) to the XbaI insert. After bacterial transformation and plasmid DNA isolation, correct insert orientation was verified by restriction using BamHI and NdeI restriction enzymes (Roche Applied Science).

The Elf-3 dominant negative mutant was obtained by subcloning Elf-3 DNA binding domain (Elf-3-DBD) into the pGEM-T as follows: the Elf-3-DBD (450 bp) fragment was obtained by PCR using Taq polymerase (AmpliTaq Gold, Life Technologies), piRES-puro-Elf-3 (Addgene, www.addgene.org) as a template and EcoRI-containing forward primer: GGGAATCGATGGTTTTCGTGACTGC,

and XbaI-containing reverse primer: GGTCTAGATCAGTTCCGACTCTGGAG. Positive clones were analyzed by restriction. pGEM-T Elf-3-DBD was digested with EcoRI and XbaI, cloned into the pEGFP-C2 plasmid (Clontech Laboratories, Palo Alto CA, USA) carrying a kanamycin resistance and verified by automated sequencing. During the cloning steps a punctual missense mutation was introduced. This mutation was reverted to the wild type sequence by site-directed mutagenesis (QuikChange, Stratagene) using the following primers: CGTGACTGCAAGaAGGGGGATCCCAAGC and GCTTGGGATCCCCCTtCTTGCAGTCACG and verified by automated sequencing.

The luciferase reporter vector containing the human 3.9 kb of COX-2 promoter (pGL3-COX-2P) and the human 3'-UTR of COX-2 (from 3074 to 3236) (pGL3P-UTR2) were kindly provided by Dr. W. Eberhardt (Klinikum der Johann Wolfgang Goethe-Universitat Frankfurt am Main, Germany, Doller et al., 2008)

2,700 bp of the rat NOX-1 promoter (-2547 +125) called pGL3/3 were kindly provided by Dr. J. Pfeilschifter (University of Frankfurt Medical School, Germany) and cloned as described previously (Plesková et al., 2006).

The luciferase reporter vector containing NOX-4 promoter and the deletion mutants of human NOX-4 promoter were kindly provided by Dr. A. Manea (Institute of Cellular Biology and Pathology "Nicolae Simionescu", Romania, Manea et al., 2010a).

630 bp of human NOX-1 3'UTR pGL3 control was purchased from Genewiz Inc. (South Plainfield, NJ, USA). Briefly, NOX-1 3'UTR was cloned in the XbaI site of the pGL3 control checking the orientation.

Human NOX-4/EGFP were kindly provided by Dr. L. Terada (University of Texas Southwestern Medical Center, Dallas, Texas, USA) and cloned as described previously (Wu et al., 2010).

Egr-1 expression was accomplished using Flag-tagged Egr-1 cDNA cloned in pcDNA3.1/Zeo+.

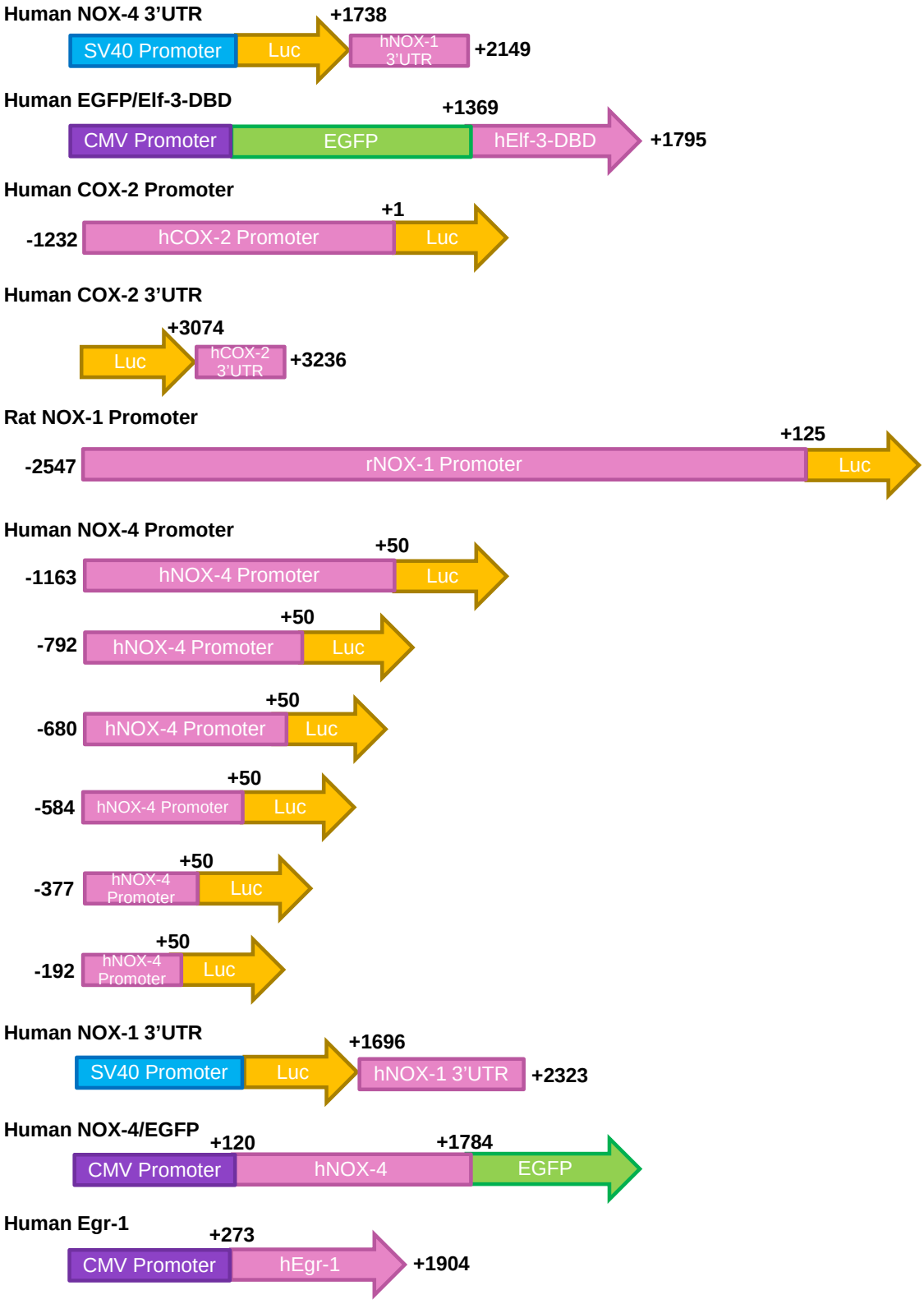

Figure 31. Scheme of the constructs. *The sequences of interest cloned in the vectors are shown in pink. Nucleotide number is also indicated. Luciferase gene (Luc) (orange), SV40 promoter (blue), cytomegalovirus (CMV) promoter (purple) and EGFP (green) are also shown.*

8. DNA AND siRNA TRANSFECTIONS

Cells were seeded in 12-well culture plates at a density of 6×10^4 cells per well and incubated overnight. Next day, the medium was replaced with DMEM-F12 with 10% FBS and glutamine without antibiotics because of their toxicity in the presence of liposomes. Then cells were transiently transfected with 0.5 µg luciferase reporter gene construct per well using the Lipofectamine LTX Plus reagent prepared in Opti-Mem (Life Technologies) in a ratio Plus:LTX 1:2.5. After 6 h, media was changed and cells were transferred to depletion medium overnight. After stimulation, cells were washed with PBS and harvested in 80 µL of reporter lysis buffer. Luciferase activities of 20 µL of cell lysate were measured using a luminometer (Orion I, Berthold detection systems, Oak Ridge, TN, USA) according to the manufacturer's instructions. Relative luciferase activities were normalized to total protein. Variations of activity were calculated as fold increase over controls or over controls with the corresponding inhibitor.

Cells seeded in 6-well plates or transwells at a density of 1.2×10^5 or 3×10^4 cells per well, respectively, were transfected with a predesigned siRNA against HuR or a negative control siRNA (Life Technologies) using the Lipofectamine LTX Plus reagent (Life Technologies) as described above. Cells were transfected for 48 h with 20 nmol/L siRNA and silencing efficiency was determined by qPCR or western blot.

9. CELL VIABILITY ASSAY

Cell viability was assessed using the CellTiter 96 Non-Radioactive Cell Proliferation Assay MTT (Promega). This assay is a colorimetric method based on the reduction of a tetrazolium salt in formazan using the cellular NADPH or NADH. The formazan product is insoluble in culture medium and precipitates, and it is directly proportional to the number of living cells in culture.

8×10^3 cells were seeded on 96-well plates in DMEM-F12 medium and were allowed to attach for 24-48 h. After stimulation, cell survival was quantified by adding MTT tetrazolium solution (10 µL per well). After 4 hours of incubation at 37 ºC, medium was removed and 50 µL of 0.1 N HCl in isopropanol were added and mixed to dissolve the salts. Absorbance was measured at 540 nm in an

ELx800 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). The experiments were performed in duplicates. Blank was subtracted. Variations of viability were calculated as fold increase over controls.

10. CELL MIGRATION ASSAYS

VSMC migration was examined using a 6.5 mm Transwell chamber with an 8 µm pore size (Corning Costar Inc., Corning, New York, NY, USA). 3×10^4 cells were resuspended in 100 µL of medium with 10% FBS and were seeded in the upper compartment. The lower compartment of the transwell was filled with 600 µL of the same medium. Next day, medium were removed and changed by serum free medium in both compartments maintaining the same volumes (Figure 32).

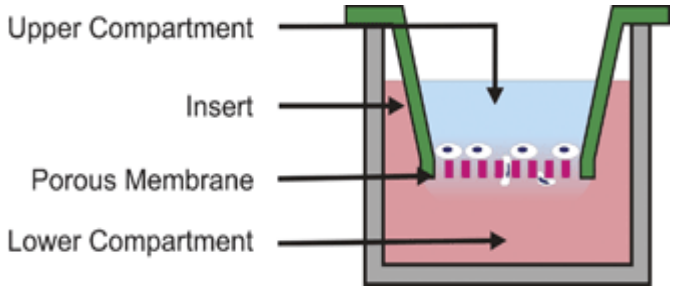

Figure 32. Transwell Assay. *Cells are seeded on a porous membrane and the chemoattractant solution is placed in the lower compartment where it diffuses into the upper compartment forming a gradient across the membrane. Cells respond by migrating through the membrane to the bottom surface where they can be subsequently fixed, stained and counted. Taken from Keenan and Folch, 2008.*

24 h later cells were pretreated or transfected in the upper compartment and then stimuli were added in the bottom compartment. Cells were allowed to migrate 5 or 24 h. Afterwards, cells of the upper membrane surface were removed with a cotton swab and the membrane was washed with PBS, fixed in 4% paraformaldehyde (PFA) during 10 min and washed twice in PBS. Hoechst 33342 (10 µg/mL) was used to stain the nuclei of the migrating cells. Membranes were cut and put over a slide with anti-fading mounting medium and sealed with nail polish. Migration values were determined by counting three fields per chamber in a confocal Leica TCS SP2 with a 40X objective and calculated as fold increase over controls.

Cell migration in response to physical damage was determined using a wound healing assay. Cells were seeded in a 24-well plate and when they reached 90% of confluence, they were starved 24 h. VSMC monolayers were wounded using a sterile 10 µL pipette tip. Phase contrast images were taken immediately (0 h) and 24 h after wounding using a Nikon microscope (Tokyo, Japan) connected to a video camera (Sony Corporation, Tokyo, Japan). To measure migration, wound area was quantified using Adobe Photoshop and expressed as percentage of wound closure over controls or over controls with the corresponding inhibitor.

11. IMMUNOFLUORESCENCE MICROSCOPY

Cells were seeded in coverslips in the well. At 60% of confluence, cells were serum starved overnight and then stimulated 24 h. Cells were rinsed with PBS, fixed with 4% paraformaldehyde (10 min), permeabilized with 0.2% Triton-PBS, blocked with 3% BSA in PBS (1 h) and incubated overnight at 4°C with anti-HuR monoclonal primary antibody diluted 1:200 in blocking solution. The fluorescein isothiocyanate–conjugated secondary IgG antibody was incubated 2 h at room temperature (1:200; Jackson ImmunoResearch, West Grove, PA, USA). After washing, coverslips were incubated 10 min with Hoechst 33342 (Life Technologies) mounted on slides and examined using an Evos XL Cell Imaging System with a 40X objective. A negative control using only the secondary antibody was used to adjust the fluorescence. Images were processed using Adobe Photoshop CS3 software (Adobe Systems Inc., San Jose, CA).

For tissue immunofluorescence, aortic segments of control and AngII-infused mice were fixed with 4% PFA for 1 h. After three washes of 10 min with PBS, arteries were incubated 30 min in a sucrose solution (0.1 M PBS and 30% sucrose), immersed in OCT tissue-tek mounting medium (Sakura finetek Europe B. V, Flemingweg, Netherland), frozen in liquid nitrogen and stored at -80 ºC. Fixed aortas were cut in cross sections of 14 µm in a cryostat and mounted in gelatinized slides. Then, slides were air-dried 90 min, blocked 1 h at 37 ºC in 0.1 M PBS containing 5% BSA and 0.4 % Triton X100 and incubated 2 h with a mouse anti-HuR (Santa Cruz Biotechnology Inc) diluted 1:100 in 0.1 M

PBS containing 0.3 % Triton X100 and 2% BSA. After washing, slides were incubated with a mouse Cy™3-conjugated secondary IgG (1:200; Jackson ImmunoResearch). All incubations were done in a humid chamber. Pictures were taken in a Leica TCS SP2 with a 63X objective. The specificity of the immunostaining was evaluated by omission of the primary antibody and processed as above.

12. HISTOLOGICAL ANALYSIS

14-µm cross sections from fixed aortas were stained with hematoxylin-eosin. All images were acquired at room temperature using a microscope (DM2000; Leica) with 10X objective. Morphometric determinations of the lumen and vessel areas were performed by using Metamorph image analysis software (Molecular Devices, Downington, PA, USA). All microscopic images of the sections were traced for the calculations of the areas. To determine the luminal area, the cross-sectional area enclosed by the internal elastic lamina was corrected to a circle by applying the form factor $l^2/4\pi$ to the measurement of the internal elastic lamina, where l is the length of the lamina. Vessel area was determined by the cross-sectional area enclosed by the external elastic lamina corrected to a circle, applying the same form factor ($l^2/4\pi$) to the measurement of the external elastic lamina. The media area was calculated as the difference between the corrected vessel and luminal areas. Internal and external diameters were calculated from luminal and vessel areas, respectively. This method avoids miscalculations of areas caused by eventual collapse of the immersion-fixed arteries.

13. IMMUNOHISTOCHEMISTRY

Mouse aortas were perfused with PBS and fixed in 4% PFA for 24 h, embedded in paraffin and prepared in 5-14 µm cross sections with a microtome (Jung RM 2055, Leica). Sections from aorta were deparaffinized in xylene and rehydrated in graded ethanol solutions. Slides were then rinsed in distilled water and treated with 10% hydrogen peroxide in methanol for 30 min to remove endogenous peroxidase activity. Sections were blocked with a 10% of normal serum in PBS-Tween 0.1% for 30 min and incubated with anti-HuR (1/100), anti-COX-2 (1/100), anti-mPGES-1 (1/100), and anti-TXAS (1/50) antibodies

overnight at 4°C. After washing 3 times in PBS-Tween 0.1%, samples were incubated for 1 h with a biotinylated secondary antibody (Vector Laboratories). After rinsing 3 times in PBS, standard Vectastain (ABC) avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA) was applied, and the slides were incubated for 30 min. Color was developed using 3,3'-diaminobenzidine and sections were counterstained with haematoxylin before dehydration, clearing, and mounting. Negative controls in which the primary antibody was omitted were included to test for non-specific binding.

14. NADPH OXIDASE ACTIVITY

The $O_2^{\bullet-}$ production generated by NADPH oxidase activity was determined by a chemiluminescence assay using lucigenin. Lucigenin is an aromatic compound, which can be reduced by $O_2^{\bullet-}$ producing light. This light is read by a luminometer.

Cells were rinsed with PBS and harvested in phosphate buffer pH 7.4 (50 mmol/L KH_2PO_4, 1 mmol/L EGTA, 150 mmol/L sucrose). The $O_2^{\bullet-}$ production generated by NADPH oxidase activity was determined by a chemiluminescence assay using lucigenin (5 µmol/L; Sigma-Aldrich) and NADPH (100 µmol/L; Sigma-Aldrich). The reaction was started by the addition of a lucigenin/NADPH mixture to the protein sample in a final volume of 250 µL. Chemiluminescence was determined every 2.4 seconds for 5 min in a plate luminometer (Berthold Detection System, Sirius, Pforzheim, Germany). Basal activity was subtracted from each reading. Luminescence was normalized by protein concentration measured by the Lowry assay and data were expressed as fold increase over the control or over controls with the corresponding inhibitor.

15. DETECTION OF REACTIVE OXYGEN SPECIES BY FLUORESCENCE MICROSCOPY

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate ROS production *in situ* in VSMC. Hydroethidine freely permeates cells and is oxidized in the presence of ROS to ethidium bromide, which is retained in the nucleus by intercalation with DNA. Ethidium bromide is excited at 535 nm and emitted at 610 nm.

VSMC were plated onto glass coverslips inserted into 6-well plates and cultured. When the cells reached 60-80% they were stimulated. Afterwards, cells were loaded with DHE (10 μmol/L; Sigma-Aldrich) in cell culture medium for 30 min at 37ºC and then rinsed with serum-free medium. Coverslips were mounted on a slide and visualized with a confocal microscope (Leica TCS SP2 with a 40X objective) using the same imaging settings in all experimental conditions. Fluorescence intensity was measured using Metamorph image analysis software (Molecular Devices). Variations of fluorescence intensity were calculated as fold increase over controls or over controls with the corresponding inhibitor.

16. H_2O_2 RELEASE

The Amplex Red reagent in combination with horseradish peroxidase (HRP), has been used to detect H_2O_2 released from biological samples. In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. Resorufin has excitation and emission maxima of approximately 571 nm and 585 nm respectively and the signal can be measured fluorometrically or spectrophotometrically.

Cells were seeded in 12-well plate, transfected with NOX-4/EGFP, EGFP alone or without transfection and then stimulated 24 h. In order to prevent interference with the resorufin measurement, we used phenol red-free medium. Supernatants were used to determine H_2O_2 release and cell lysates to measure total protein content. Amplex Red (100 μmol/L; Sigma-Aldrich) and horseradish peroxidase type II (0.2 U/mL; Sigma-Aldrich) were added to 50 μL of supernatants. Fluorescence readings were made in duplicate in a 96-well plate at Ex/Em = 530/580 nm. H_2O_2 concentration was estimated using a standard curve between 0-4.8 μmol/L of H_2O_2. Total protein of cell lysates as well as the volume of the supernatants was measured in order to normalize H_2O_2 values.

17. REAGENTS

Acrylamide (N,N'-Methylenebisacrylamide) (Bio-Rad)

Actinomycin D (Sigma-Aldrich)

Agarose (Bio-Rad)

Angiotensin II human acetate (Sigma-Aldrich)

BSA (Bovine serum albumin) (Sigma-Aldrich)

Celecoxib (generously provided by Pfizer Inc)

Chelerytrhine (Tocris, Biogen Científica, Spain)

Chloroform (Merck)

Cycloheximide (4-[(2R)-2-[(1S,3S,5S)-3,5-Dimethyl-2-oxocyclohexyl]-2-hydroxyethyl]piperidine-2,6-dione) (Sigma-Aldrich)

DEPC (Diethylpyrocarbonate) (Sigma-Aldrich)

DMSO (Dimethyl sulfoxide) (Merck)

DTT (Dithiothreitol) (Promega)

EDTA (Ethylenediaminetetraacetic acid) (Sigma-Aldrich)

EGTA (ethylene glycol tetraacetic acid) (Sigma-Aldrich)

Ethanol (Merck)

Furegrelate 5-(3-pyridinylmethyl)-2-benzofurancarboxylic acid, sodium salt) (Cayman Chemical)

Glycine (Sigma-Aldrich)

Glycerol (Panreac, Castellar del Vallés, Barcelona, España)

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma-Aldrich)

Interleukin 1β mouse (Sigma-Aldrich)

Isopropanol (Merck)

KN93 (N-[2-[N-(4-Chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide phosphate salt) (Calbiochem, Darmstadt, Germany)

L798106 (N-[(5-Bromo-2-methoxyphenyl)sulfonyl]-3-[2-(2-naphthalenylmethyl)phenyl]-2-propenamide) (Sigma-Aldrich)

LY294002 (2-Morpholin-4-yl-8-phenylchromen-4-one) (Calbiochem)

Magnesium chloride (Panreac)

ML171 (2-Acetylphenothiazine) (Sigma-Aldrich)

MS-444 (generously provided by Novartis, Basil, Switzerland)

NP-40 (Igepal) (Octylphenoxy)polyethoxyethanol) (Sigma-Aldrich)

Ozagrel ((2E)-3-[4-(1H-imidazol-1-ylmethyl)phenyl]acrylic acid) (generously provided by Kissei Pharmaceutical CO, Matsumoto, Japan)

Paraformaldehyde (Panreac)

PD98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) (AlomoneLabs)

PMSF (phenylmethanesulfonyl fluoride) (Sigma-Aldrich)

Potassium Chloride (Panreac)

Potassium hydrogen phosphate (Panreac)

RNAase inhibitor (Promega, Mannheim, Germany)

Rofecoxib (4-(4-methylsulfonylphenyl)-3-phenyl-5H-furan-2-one) (LKT Laboratories Inc. St Paul, MN, USA)

SB203580 (4-(4'-Fluorophenyl)-2-(4'-methylsulfinylphenyl)-5-(4'-pyridyl)-imidazole) (Calbiochem)

SC19220 (2-Acetylhydrazide 10(11H)-carboxylic acid) (Sigma-Aldrich)

Sodium chloride (Panreac)

Sodium deoxicholate (Sigma-Aldrich)

Sodium fluoride (Sigma-Aldrich)

Sodium orthovanadate (Sigma-Aldrich)

SP600125 (1,9-Pyrazoloanthrone, Anthrapyrazolone) (Calbiochem)

SQ29548 ([1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid) (ICN Iberica, Barcelona, Spain)

Suberoylanilide hydroxamic acid (N-hydroxy-N'-phenyl-octanediamide) (Sigma-Aldrich)

Sucrose (Merck)

SDS (sodium dodecyl sulfate) (Bio-Rad)

Tris (tris(hydroxymethyl)aminomethane) (Bio-Rad)

Triton X100 (Sigma-Aldrich)

tRNA (Promega)

Tyrphostin AG1478 (N-(3-Chlorophenyl)-6,7-dimethoxy-4-quinazolinamine) (AlomoneLabs, Jerusalem, Israel)

Trichostatin (7-[4-(dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxohepta-2,4-dienamide) (Sigma-Aldrich)

Tween-20 (Bio-Rad)

U0126 (1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio)butadiene) (Calbiochem, Darmstadt, Germany)

U46619 ((Z)-7-[(1S,4R,5R,6S)-5-[(E,3S)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid) (Cayman Chemical)
W7 (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide) (Calbiochem)
W12 (N-(4-aminobutyl)-1-naphthalenesulfonamide) (Calbiochem)
β-glycerophosphate (Sigma-Aldrich)
β-mercaptoethanol (Bio-Rad)
5-aza-2'-deoxycytidine (2′-Deoxy-5-azacytidine, 4-Amino-1-(2-deoxy-β-D-ribofuranosyl)-1,3,5-triazin-2(1H)-one) (Sigma-Aldrich)
16,16-dimethyl Prostaglandin E_2) (Calbiochem)

General reagents of the laboratory were purchased from different providers such as Sigma-Aldrich or Merck.

18. DATA ANALYSIS AND STATISTICS

Data analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). All values are expressed as mean ± SEM of the animals used in each experiment or independent cell culture-based experiments. Statistical analysis was done by Student's *t* test, Mann-Whitney test or by one-way ANOVA followed by a Bonferroni test. Values were considered to be significant when $P<0.05$.

Results

1. HuR MEDIATES THE SYNERGISTIC EFFECTS OF ANGII AND IL-1β ON VASCULAR COX-2 EXPRESSION AND CELL MIGRATION

1.1. ANGII AND IL-1β SYNERGISTICALLY INDUCE COX-2 EXPRESSION IN VSMC THROUGH ERK1/2 PATHWAY

In basal conditions, rat VSMC express low-to-undetectable COX-2 mRNA and protein levels and after 24 h stimulation with either AngII (0.1 μmol/L) or IL-1β (10 ng/mL), a modest increase in COX-2 expression was observed (Figure 33). However, co-stimulation of rat VSMC with AngII and IL-1β led to a synergistic increase in COX-2 mRNA and protein levels (Figure 33).

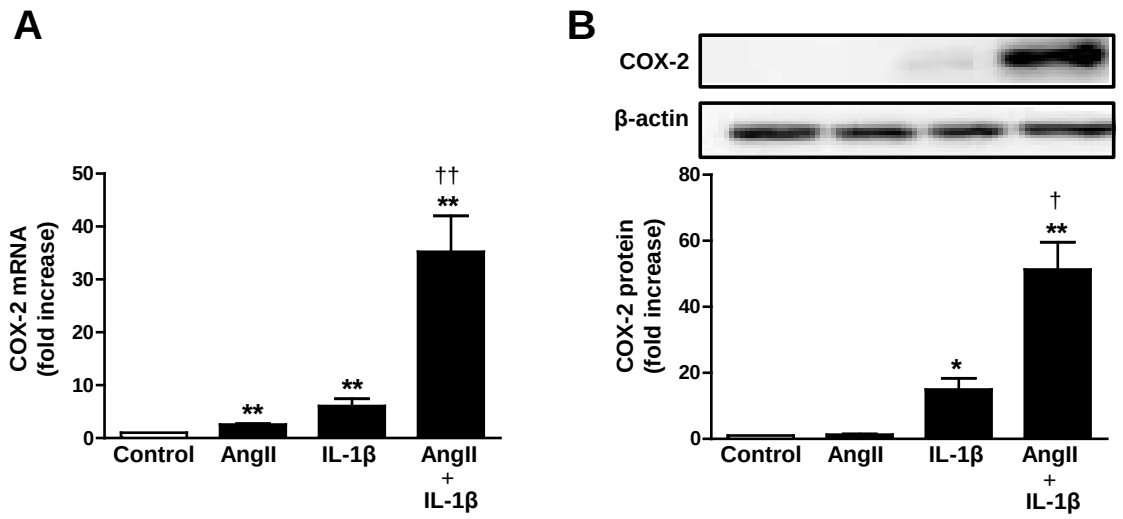

Figure 33. AngII potentiates IL-1β-induced COX-2 expression in rat VSMC. *Effect of AngII, IL-1β and AngII+IL-1β (24 h) on COX-2 mRNA levels* ***(A)*** *and protein expression* ***(B)*** *in rat VSMC. Data are expressed as mean ± SEM.* $^{*}p<0.05$, $^{**}P<0.01$ *vs Control;* $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ *vs AngII or IL-1β. n=4-9.*

The exacerbated induction of COX-2 mRNA in the presence of AngII+IL-1β was observed to occur as early as 1 h after stimulation and persisted out to 24 h with enhanced COX-2 protein expression being observed at 24 h (Figure 34).

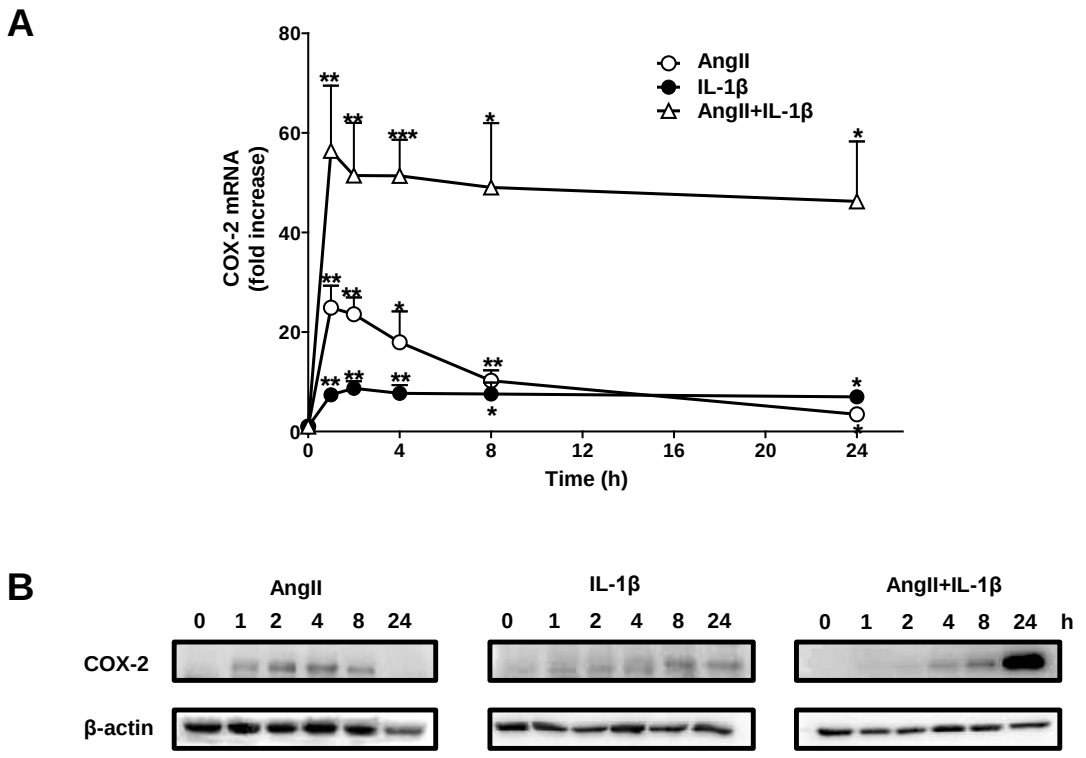

Figure 34. Effect of AngII, IL-1β and AngII+IL-1β on COX-2 expression. *Time course of COX-2 mRNA levels* ***(A)*** *and protein expression* ***(B)*** *in rat VSMC. Data are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs unstimulated cells. n=5-7.*

Similar effects of AngII, IL-1β and AngII+IL-1β on COX-2 expression were observed in human VSMC indicating that this effect is conserved and adding to this finding an additional relevance (Figure 35).

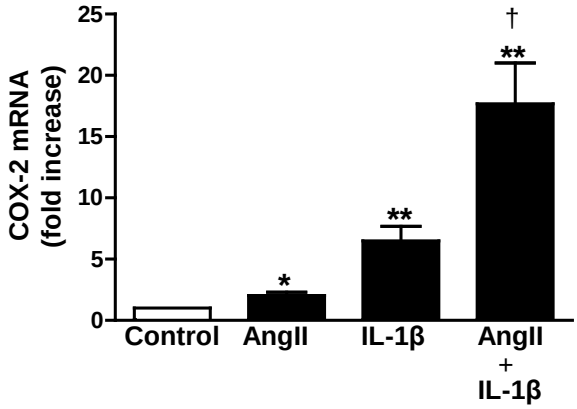

Figure 35. AngII potentiates IL-1β-induced COX-2 expression in human VSMC. *Effect of AngII, IL-1β or AngII+IL-1β (24 h) on COX-2 mRNA levels in human VSMC. Data are expressed as mean ± SEM. *P<0.05, **P<0.01; †P<0.05 vs AngII or IL-1β. n=6-7.*

MAPK pathways are involved in COX-2 expression induced by AngII in different cell types (Ohnaka et al., 2000; Pham et al., 2008; Galán et al., 2011). AngII also activates Akt pathway in VSMC (Takahashi et al., 1999). Therefore, we analyzed the involvement of these signaling pathways in the synergistic effect of AngII and IL-1β on COX-2 expression. As expected, AngII, IL-1β and AngII+IL-1β promoted rapid phosphorylation of ERK1/2, JNK, p38 MAPK and Akt (Figure 36).

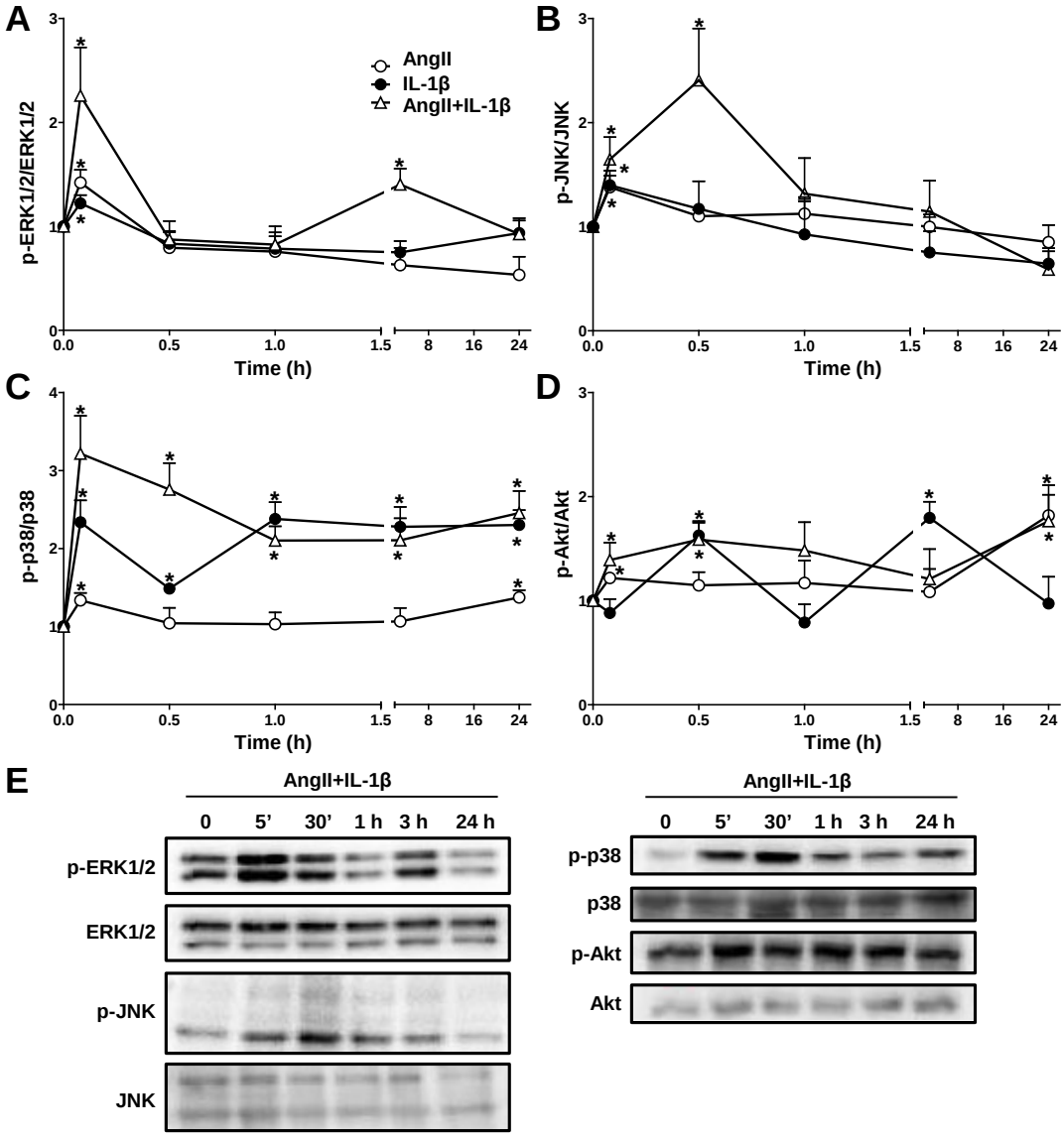

Figure 36. Effect of AngII, IL-1β and AngII+IL-1β on ERK1/2, JNK, p38 MAPK and Akt activation. *Quantification of the time course activation of ERK1/2* ***(A)****, JNK* ***(B)*** *and p38* ***(C)*** *MAPK and Akt* ***(D)*** *in response to AngII, IL-1β and AngII+IL-1β in rat VSMC.* ***(E)*** *representative blots of MAPK and Akt activation in response to AngII+IL-1β. Data are expressed as mean ± SEM.* $^{*}P<0.05$ *vs unstimulated cells. n=4-9.*

Interestingly, the ERK1/2 inhibitor (U0126, 10 μmol/L) decreased the AngII+IL-1β-induced COX-2 mRNA expression, whereas the JNK (SP600125, 20 μmol/L), p38 MAPK (SB203580, 10 μmol/L) and PI3K (LY294002, 10 μmol/L) inhibitors did not (Figure 37). These results suggest that ERK1/2 is implicated in AngII+IL-1β-induced COX-2 expression.

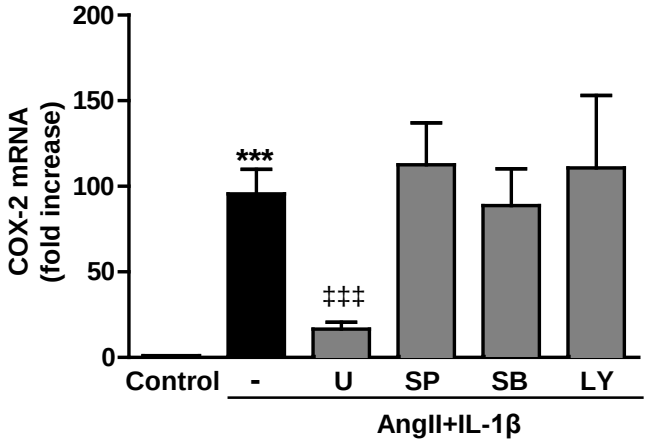

Figure 37. AngII potentiates IL-1β-induced COX-2 expression in VSMC through ERK1/2. *Effect of MAPKs inhibitors (U: U0126; SP: SP600125; SB: SB203580) and PI3K inhibitor (LY: LY294002) on COX-2 mRNA levels induced by AngII+IL-1β (4 h). Data are expressed as mean ± SEM. ***P<0.001 vs Control; ‡‡‡P<0.001 vs AngII+IL-1β. n=4-9.*

1.2. HuR IS INVOLVED IN THE COX-2 mRNA STABILIZATION INDUCED BY ANGII IN VSMC

COX-2 expression is regulated by both transcriptional and post-transcriptional mechanisms (Harper and Tyson-Capper, 2008). Previous studies in intestinal epithelial cells, human mesangial cells and adventitial fibroblasts demonstrated transcriptional activation of COX-2 expression and changes in COX-2 mRNA stability in response to AngII (Pham et al., 2008; Doller et al., 2008; Galán et al., 2011). To determine whether the effect of AngII on IL-1β-induced COX-2 expression was due to changes in transcription, COX-2 transcriptional activity was measured in VSMC transfected with a reporter plasmid containing the human COX-2 promoter fused to luciferase cDNA. As expected, IL-1β increased COX-2 promoter activity but the combination of AngII+IL-1β resulted in a similar transcriptional activity (Figure 38).

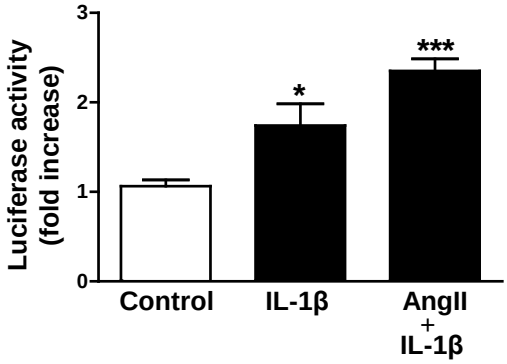

Figure 38. IL-1β and AngII+IL-1β increases COX-2 promoter activity. *Rat VSMC were transiently transfected with the luciferase reporter construct containing COX-2 promoter. Luciferase activity was assayed in untreated (control) VSMC and after incubation with AngII+IL-1β (4 h). Data are expressed as mean ± SEM. *P<0.05, ***P<0.001 vs Control. n=5-7.*

These results suggested post-transcriptional regulatory mechanisms to be the primary effector of the synergistic induction of COX-2 expression in response to AngII+IL-1β. To test this hypothesis, we performed mRNA stability assays. Figure 39A shows that AngII stabilized COX-2 mRNA in IL-1β-triggered VSMC. Accordingly, mRNA stability in AngII+IL-1β treated cells was greater than in control cells (Figure 39A). We also performed transfections of VSMC using a luciferase reporter plasmid containing a 150 bp 3'UTR from COX-2 mRNA, which included the binding site for HuR. Figure 39B shows that both stimuli were needed to increase COX-2 3'UTR luciferase activity.

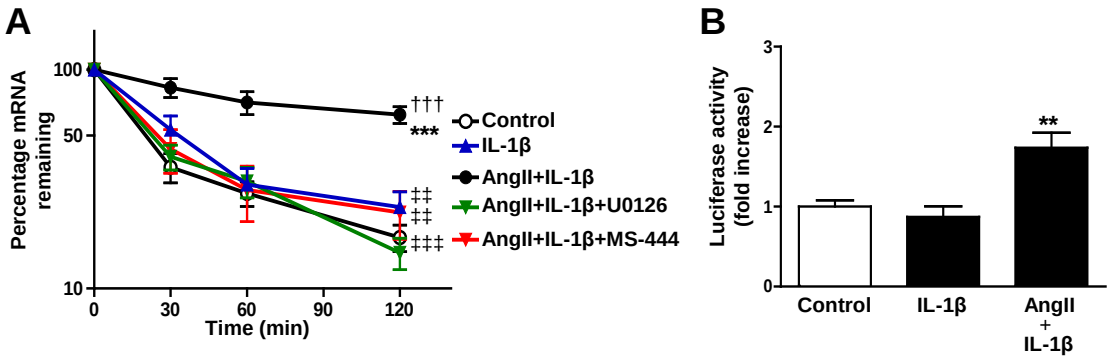

Figure 39. The synergistic effect of AngII and IL-1β on COX-2 expression in VSMC is due to an increase of COX-2 mRNA stability mediated by HuR. A, *Effect of U0126 and MS-444 on COX-2 mRNA stability in cells stimulated with AngII+IL-1β for 24 h. Effect of IL-1β is also shown. Actinomycin D was added for the indicated times and COX-2 mRNA levels were measured by qPCR.* ***B,*** *Rat VSMC were transfected with a luciferase reporter construct containing the COX-2 3'-UTR. Luciferase activity was assayed in untreated (control) VSMC and after incubation with IL-1β and AngII+IL-1β (24 h). Data are expressed as mean ± SEM. **P<0.01, ***P<0.001 vs Control; †††P<0.001 vs IL-1β; ‡‡P<0.01, ‡‡‡P<0.001 vs AngII+IL-1β. n=4-8.*

In unstressed cells, the mRNA binding protein HuR is predominantly localized to the nucleus (>90%) and can be exported to the cytoplasm to stabilize different mRNAs including COX-2 mRNA (Dixon et al., 2001; Young et al., 2012). We explored the role of HuR on COX-2 expression induced by AngII+Il-1β. As shown in Figure 40, treatment of VSMC with AngII+IL-1β increased HuR cytoplasmic localization and this effect was abolished by ERK1/2 (+PD98059, 20 μmol/L) or HuR (+MS-444, 8 μmol/L) inhibition.

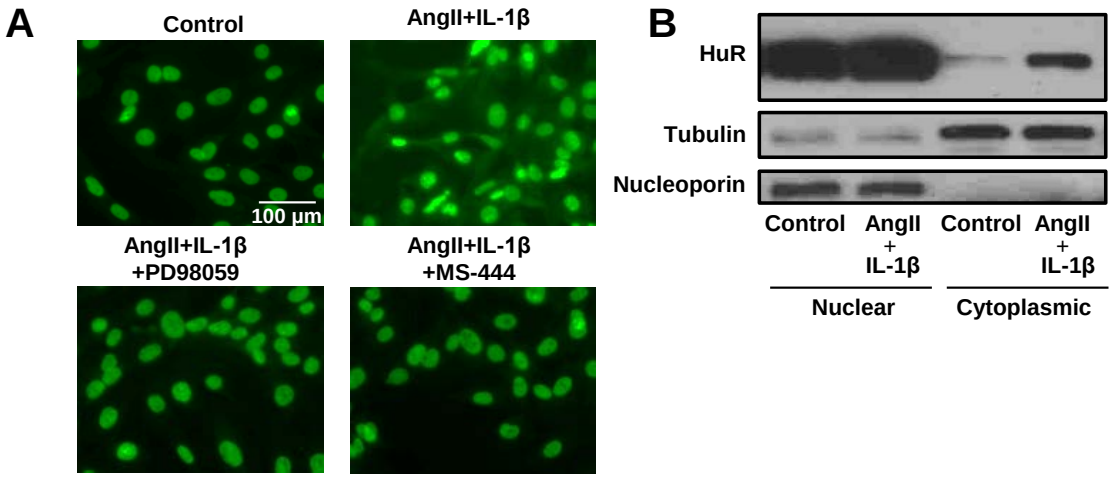

Figure 40. HuR is translocated to the cytoplasm in the presence of AngII+IL-1β. ***A,*** *Effect of PD98059 and MS-444 on HuR subcellular localization assayed by HuR immunofluorescence.* ***B,*** *Western blotting of cellular fractions from VSMC unstimulated (control) or stimulated with AngII+IL-1β (24 h). n=4-8.*

Consistent with these observations on HuR trafficking, another ERK1/2 inhibitor (U0126, 10 μmol/L) and MS-444 also abolished the increased stability of COX-2 mRNA observed in AngII+IL-1β-stimulated VSMC (Figure 39A). Moreover, MS-444 or HuR knockdown by siRNA (Figure 41A) reduced COX-2 mRNA levels induced by AngII+IL-1β (Figures 41B and 41C).

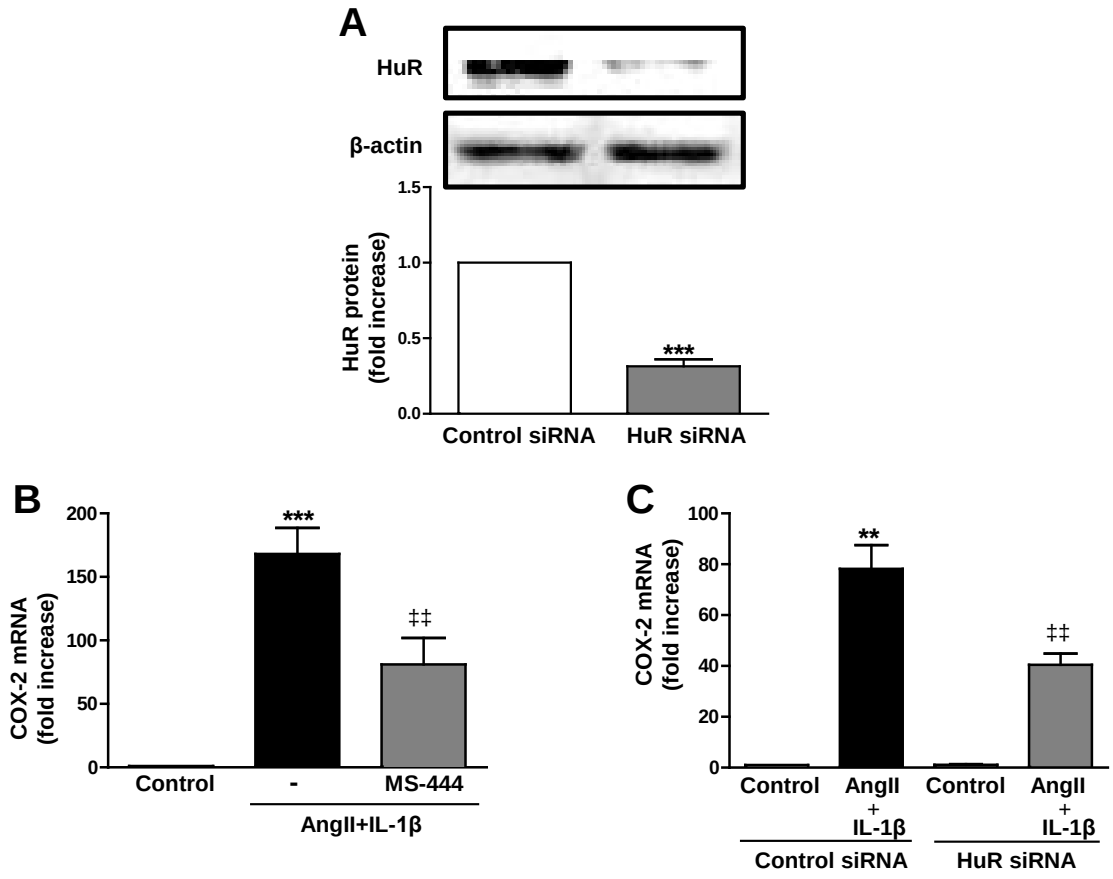

Figure 41. HuR blockade decreases COX-2 expression induced by AngII+IL-1β. A, *HuR expression in cells transfected with control or HuR siRNA. Effects of MS-444 (**B**) and HuR siRNA (**C**) on COX-2 mRNA levels in VSMC stimulated with AngII+IL-1β (24 h). Data are expressed as mean ± SEM. $^{**}P<0.01$, $^{***}P<0.001$ vs Control; $^{‡‡}P<0.01$ vs AngII+IL-1β. n=4-8.*

MS-444 was originally characterized as MLC kinase (MLCK) inhibitor in the μmol/L-range (Aotani et al., 1995). However, the concentration used in these experiments did not affect MLC phosphorylation induced by AngII+IL-1β (Figure 42A) or cell viability (Figure 42B).

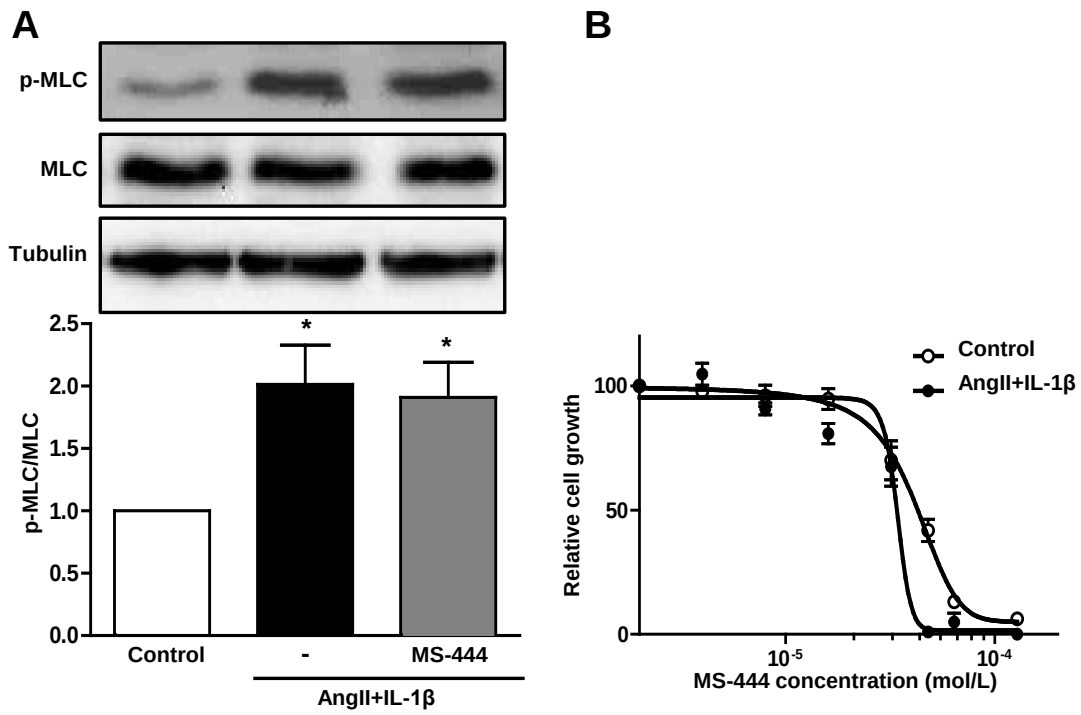

Figure 42. MS-444 does not affect myosin light chain kinase activity or cell viability of VSMC. A, *Quantification and representative blots of the effect of MS-444 on p-MLC and MLC expression in VSMC treated with AngII+IL-1β (24 h).* ***B,*** *Concentration-dependent effect of MS-444 on cell viability in control or AngII+IL-1β-treated VSMC. Data are expressed as mean ± SEM.* **P<0.05 vs Control. n=5-7.*

To determine if AngII+IL-1β signaling promotes increased cytoplasmic HuR binding to COX-2 mRNA, ribonucleoprotein immunoprecipitation was done. Cytoplasmic lysates of VSMC unstimulated or stimulated 24 h with AngII+IL-1β were immunoprecipitated using an antibody against HuR or control IgG. The association of COX-2 mRNA with HuR was assayed by qPCR of COX-2 mRNA in immunoprecipitates. We observed an enrichment in COX-2 mRNA in anti-HuR precipitates from cells treated with AngII+IL-1β, but not in unstimulated cells or in anti-IgG precipitates (Figure 43A). GAPDH mRNA co-precipitation was used as control to assess the nonspecific background (Figure 43B).

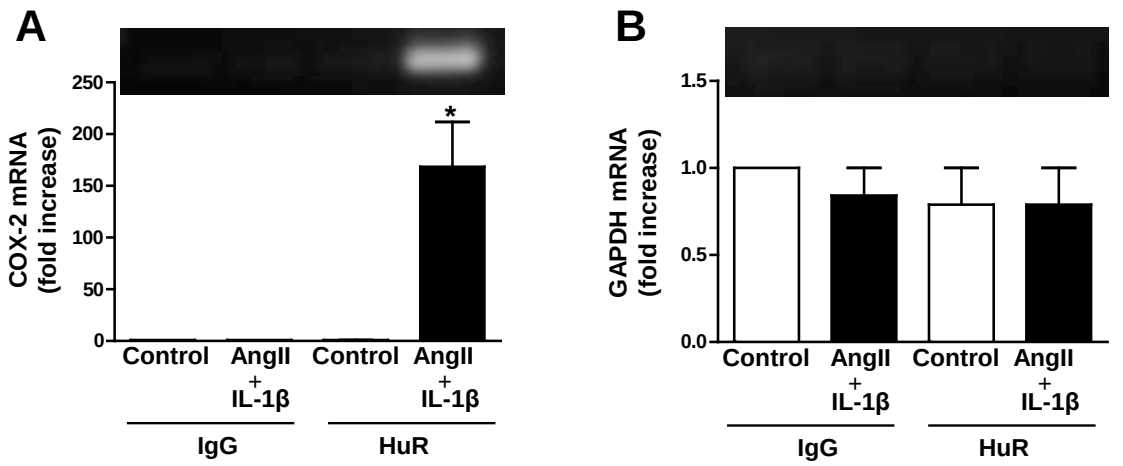

Figure 43. HuR binds to COX-2 mRNA. *Ribonucleoprotein immunoprecipitation of HuR or control IgG was done to isolate COX-2 mRNA* ***(A)*** *or GAPDH* ***(B)*** *bound to HuR in VSMC unstimulated (control) or stimulated with AngII+IL-1β (24 h). Data are expressed as mean ± SEM.* $^{*}P<0.05$ *vs Control. n=4.*

All together, these findings suggest that AngII+IL-1β induce an ERK1/2-dependent activation of HuR that is responsible for increased COX-2 mRNA stability and COX-2 levels in VSMC.

1.3. EFFECT OF ANGII AND IL-1β ON mPGES-1, PGIS AND TXAS EXPRESSION IN RAT AND HUMAN VSMC

To assess whether prostanoid synthases were also regulated by AngII, IL-1β or the combination of both, we measured mPGES-1, PGIS and TXAS mRNA and protein levels. Rat VSMC express low-to-undetectable mPGES-1 mRNA and protein levels (Figure 44). IL-1β treatment led to a time-dependent increase in mPGES-1 mRNA levels, whereas AngII treatment decreased mPGES-1 mRNA; accordingly, AngII+IL-1β increased mPGES-1 mRNA levels less than IL-1β alone (Figures 44A). Similar results were observed when mPGES-1 mRNA and protein levels were examined only at 24 h stimulation (Figure 44B and 44C).

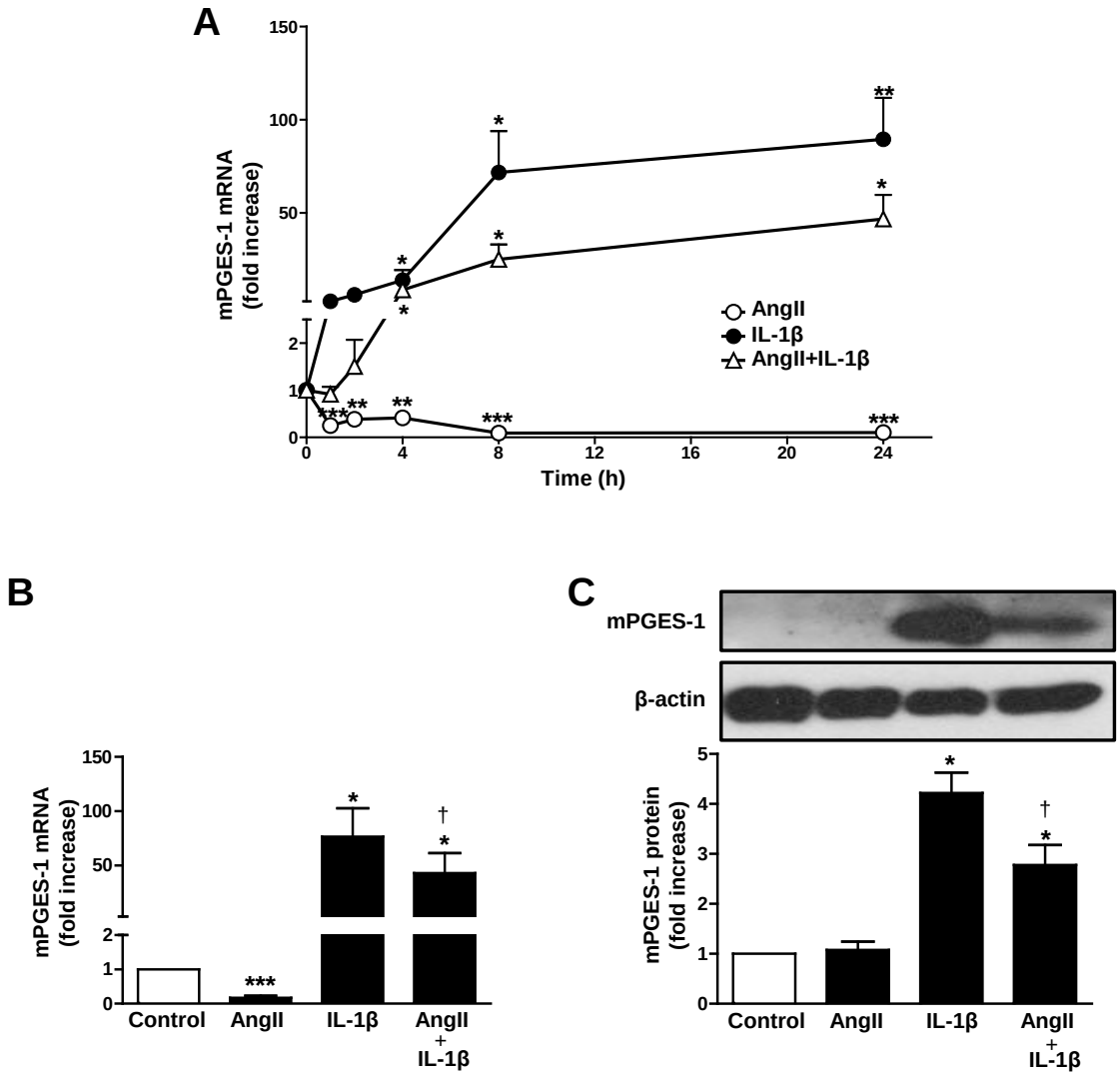

Figure 44. Effect of AngII, IL-1β and AngII+IL-1β on mPGES-1 expression. A, *Time course of mPGES-1 mRNA levels in rat VSMC stimulated with AngII, IL-1β or AngII+IL-1β. Effect of AngII, IL-1β or AngII+IL-1β (24 h) on mPGES-1 mRNA levels* ***(B)*** *and protein expression* ***(C)****. Representative blots are also shown. Data are expressed as mean ± SEM.* $^{*}P<0.05$, $^{***}P<0.001$ *vs unstimulated (control) cells;* $^{\dagger}P<0.05$ *vs AngII or IL-1β. n=4-8.*

In contrast to mPGES-1 expression, PGIS mRNA and protein levels were significantly induced by AngII and not affected by IL-1β (Figure 45); the effects of AngII+IL-1β on PGIS mRNA and protein were similar to those produced by AngII alone (Figure 45).

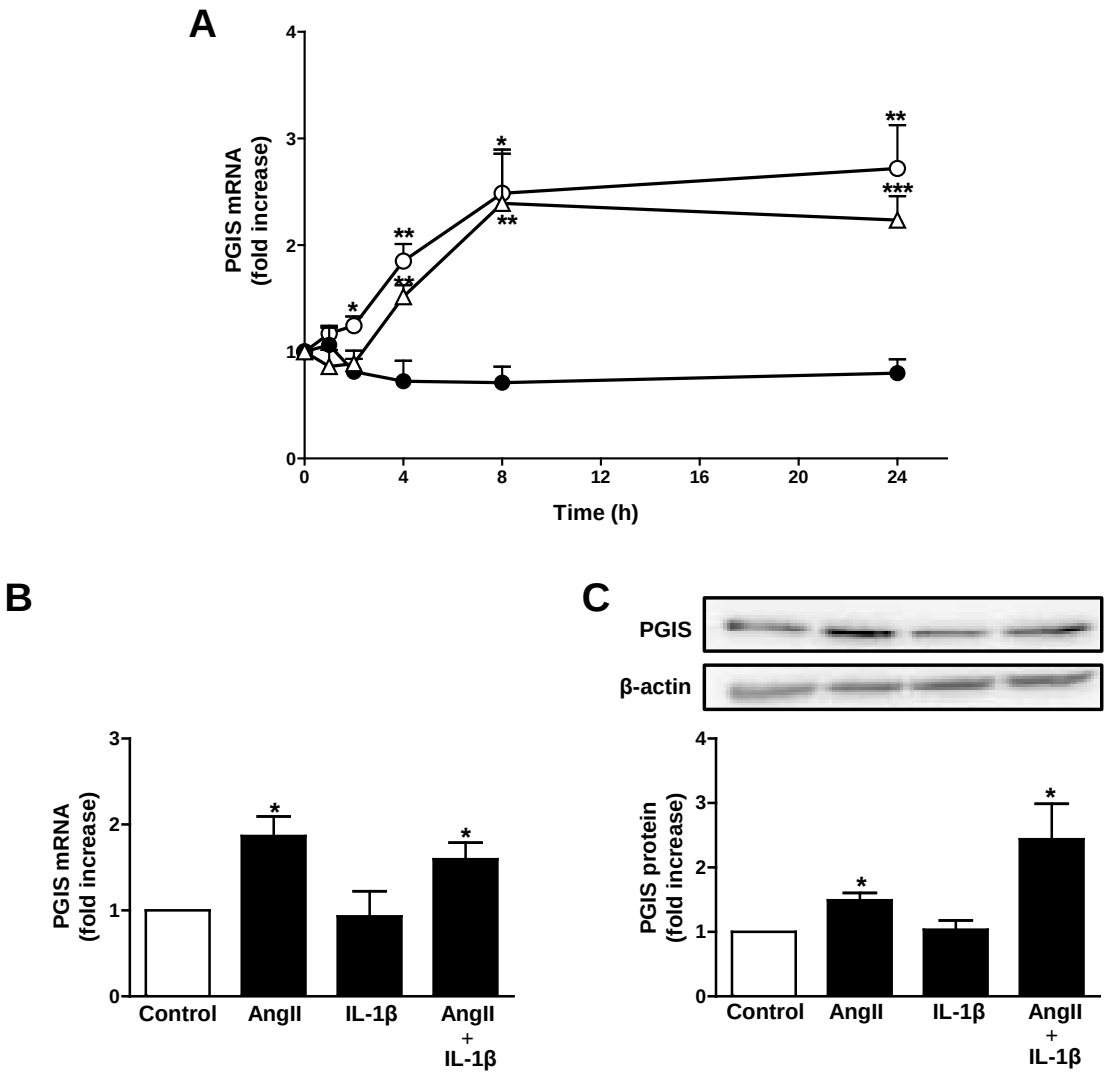

Figure 45. Effect of AngII, IL-1β and AngII+IL-1β on PGIS expression. A, *Time course of PGIS mRNA levels in rat VSMC stimulated with AngII, IL-1β or AngII+IL-1β. Effect of AngII, IL-1β or AngII+IL-1β (24 h) on PGIS mRNA levels* ***(B)*** *and protein expression* ***(C)****. Representative blots are also shown. Data are expressed as mean ± SEM.* $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs unstimulated (Control) cells. n=4-7.*

TXAS mRNA levels were increased in a time-dependent manner by IL-1β, AngII and AngII+IL-1β (Figures 46A and 46B). At protein level, all stimuli slightly but significantly increased TXAS levels (Figure 46C).

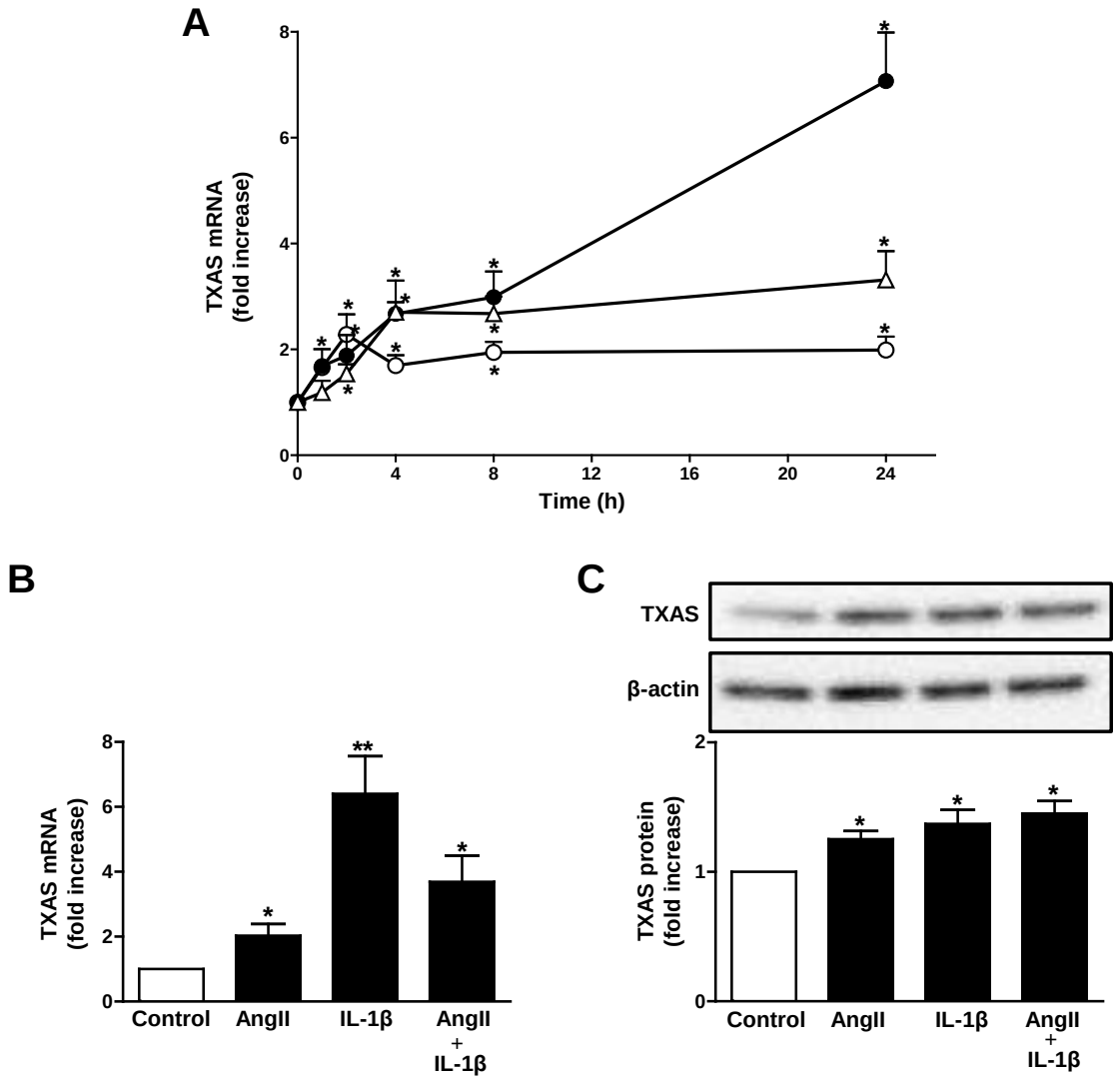

Figure 46. Effect of AngII, IL-1β and AngII+IL-1β on TXAS expression. A, *Time course of TXAS mRNA levels in rat VSMC stimulated with AngII, IL-1β or AngII+IL-1β. Effect of AngII, IL-1β or AngII+IL-1β (24 h) on TXAS mRNA levels* ***(B)*** *and protein expression* ***(C)****. Representative blots are also shown. Data are expressed as mean ± SEM.* $^{*}P<0.05$, $^{**}P<0.01$ *vs unstimulated (Control) cells. n=4-7.*

When exposed during 24 h to AngII and/or IL-1β, human VSMC showed a similar pattern that rat VSMC in mPGES-1, PGIS and TXAS mRNA levels except PGIS which was also induced by IL-1β in human cells (Figure 47).

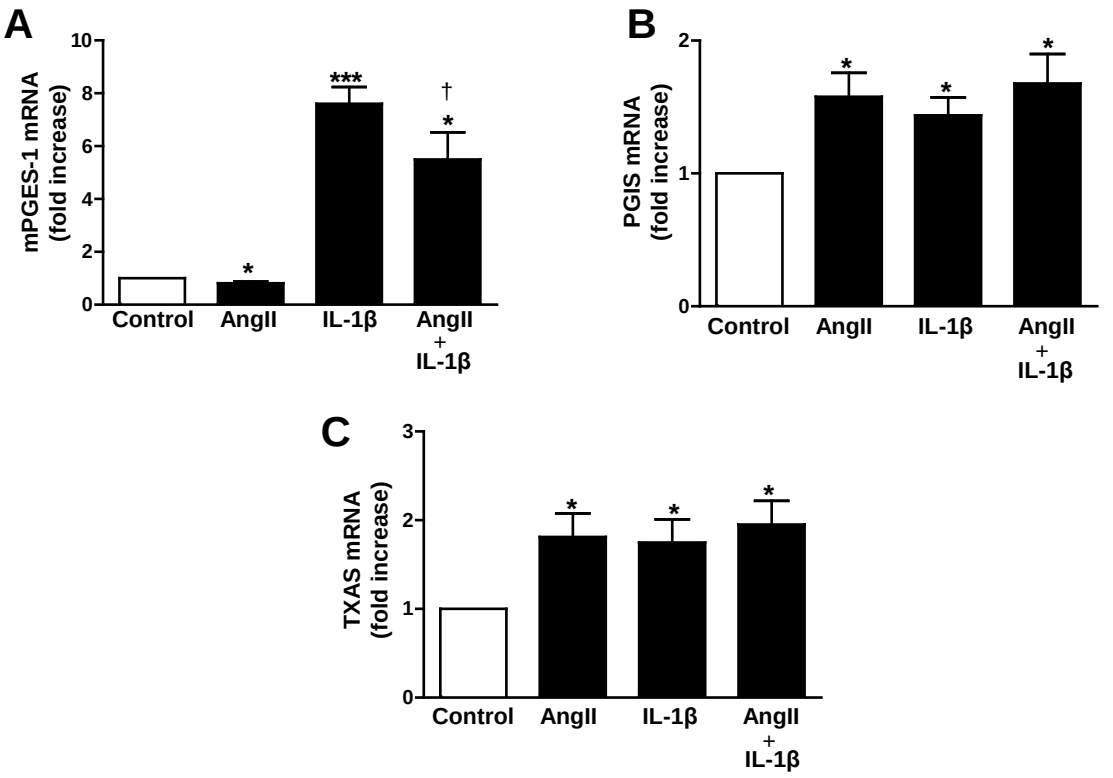

Figure 47. AngII and IL-1β regulate differentially mPGES-1, PGIS and TXAS expressions in human VSMC. *Effect of AngII, IL-1β or AngII+IL-1β (24 h) on mPGES-1* ***(A)****, PGIS* ***(B)*** *and TXAS* ***(C)*** *mRNA levels in human VSMC. Data are expressed as mean ± SEM.* **P<0.05,* ****P<0.01 vs Control;* †*P<0.05 vs AngII or IL-1β. n=6-7.*

The increased mPGES-1 mRNA levels observed after 24 h stimulation with AngII+IL-1β were blocked with ERK1/2 (U0126), JNK (SP600125), p38 MAPK (SB203580) and PI3K (LY294002) inhibitors (Figure 48A). However, PGIS mRNA levels were diminished only by the ERK1/2 inhibitor (Figure 48B). TXAS mRNA levels were reduced by ERK1/2, p38 MAPK and PI3K inhibitors (Figure 48C). These results indicate that different signaling pathways are involved in the regulation of mPGES-1, PGIS and TXAS expression.

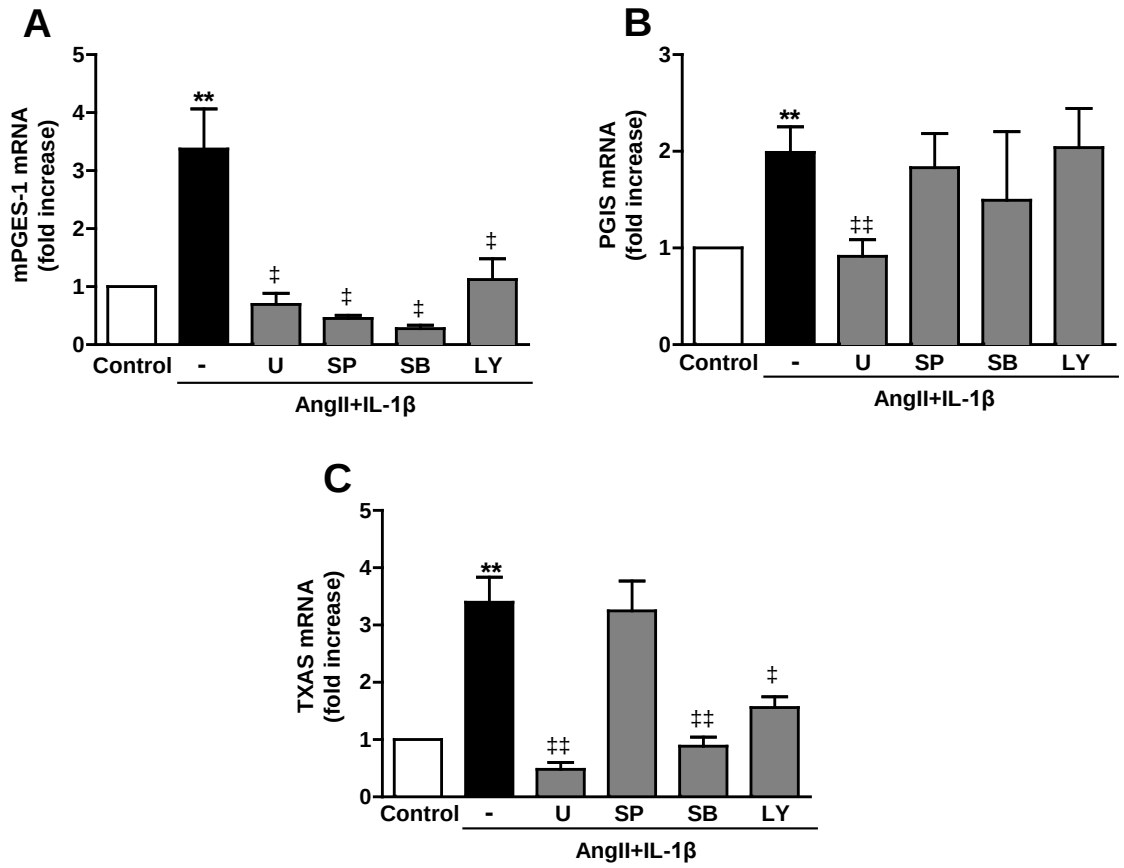

Figure 48. Different signaling pathways are involved in mPGES-1, PGIS and TXAS expression. *Effect of ERK1/2 (U0126, U), JNK (SP600125, SP), p38 MAPK (SB203580, SB) and PI3K (LY294002, LY) inhibitors on mPGES-1* ***(A)****, PGIS* ***(B)*** *and TXAS* ***(C)*** *mRNA levels in rat VSMC stimulated with AngII+IL-1β (4 h). Data are expressed as mean ± SEM.* ***P<0.01 vs Control;* ‡*P<0.05,* ‡‡*P<0.01 vs AngII+IL-1β. n=5-8.*

1.4. COX-2-DERIVED PROSTANOIDS MEDIATE THE SYNERGISTIC EFFECT OF ANGII AND IL-1β ON CELL MIGRATION

Transwell and wound healing assays were used to study cell migration in VSMC. Both AngII and IL-1β induced VSMC migration that was synergistically increased in the presence of both stimuli (Figure 49).Thus, the effect of the combination of AngII and IL-1β was greater than the sum of the effects of each stimulus alone.

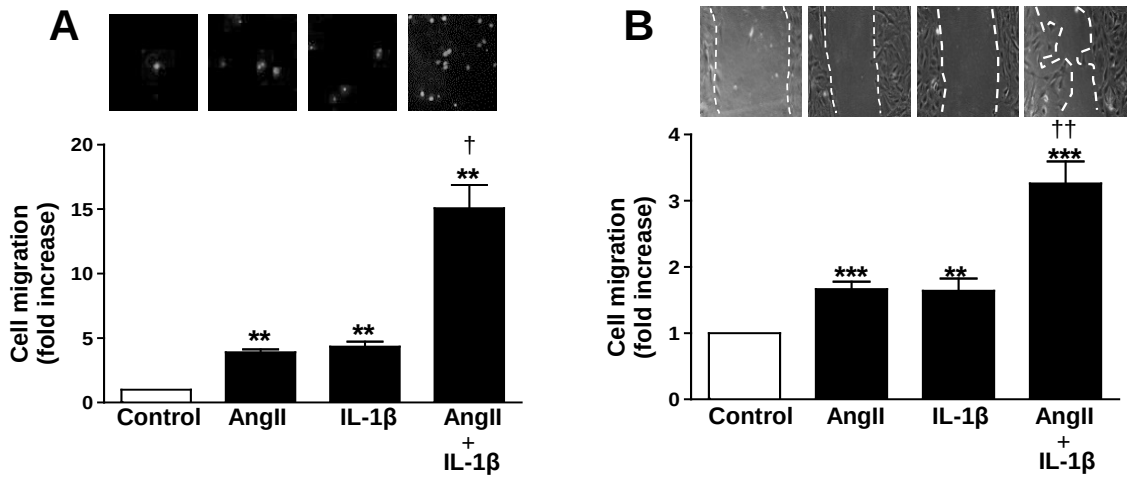

Figure 49. AngII+IL-1β potentiates cell migration. *Transwell (5 h)* ***(A)*** *and wound healing (24 h)* ***(B)*** *migration assays were performed in rat VSMC unstimulated (control) or stimulated with AngII, IL-1β or AngII+IL-1β. Data are expressed as mean ± SEM. $^{**}P<0.01$, $^{***}P<0.001$ vs Control; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs AngII or IL-1β. n=3-6.*

The effect of AngII+IL-1β on cell migration was reduced by U0126 and by inhibitors of COX-2 (celecoxib, 10 μmol/L), TXAS (furegrelate, 10 μmol/L and ozagrel, 10 μmol/L), TP receptor (SQ29548, 3 μmol/L), EP_1 receptor (SC19220, 10 μmol/L) and EP_3 receptor (L798106, 1 μmol/L) (Figure 50).

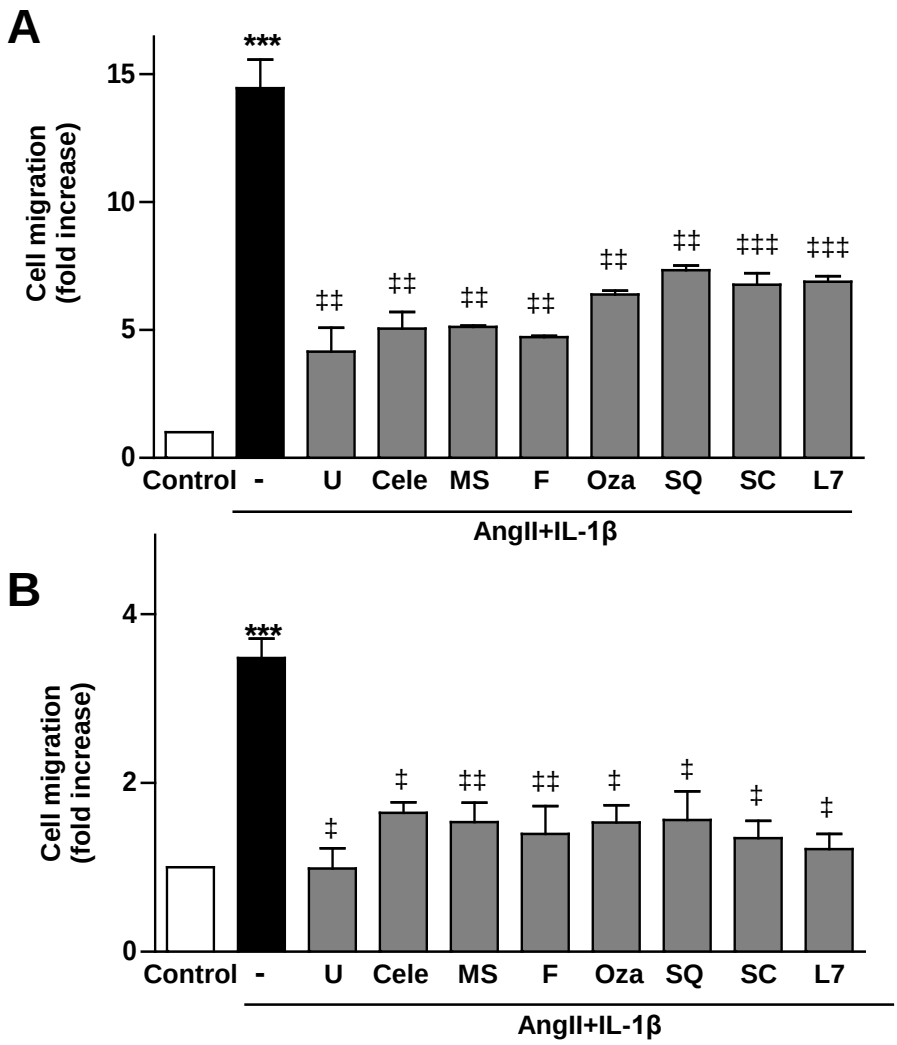

Figure 50. AngII+IL-1β potentiates cell migration through COX-2-derived PGE_2 and TXA_2. *Transwell* ***(A)*** *and wound healing* ***(B)*** *migration assays were performed in rat VSMC unstimulated (control) or stimulated with AngII+IL-1β with or without U0126 (U), celecoxib (Cele), MS-444 (MS), furegrelate (F), ozagrel (Oza), SQ29548 (SQ), SC19220 (SC) or L798106 (L7). Data are expressed as mean ± SEM. $^{***}P<0.001$ vs Control; $^{‡}P<0.05$, $^{‡‡}P<0.01$, $^{‡‡‡}P<0.01$ vs AngII+IL-1β. n=3-6.*

Since COX-2 is under the control of HuR, we investigated whether HuR blockade also affects VSMC migration. As shown in Figures 50 and 51, AngII+IL-1β-induced VSMC migration was inhibited by MS-444 and by HuR siRNA.

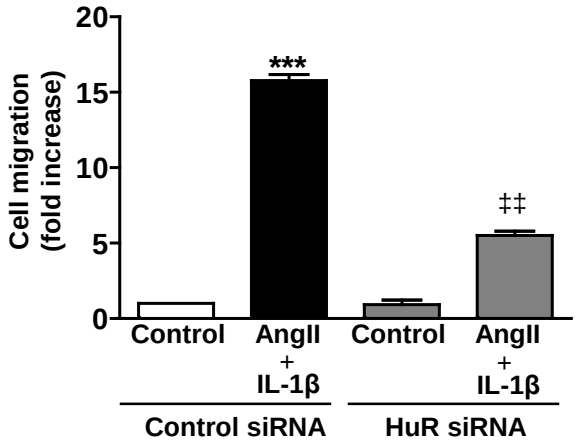

Figure 51. HuR is involved in AngII+IL-1β-induced cell migration. *Transwell migration assays were performed in cells transfected with control or HuR siRNA and stimulated or not with AngII+IL-1β. Data are expressed as mean ± SEM.* ****P<0.001 vs Control;* ‡‡*P<0.01 vs AngII+IL-1β. n=3-6.*

All together these results indicate that ERK1/2, HuR and PGE_2 and TXA_2 derived presumably from COX-2 are involved in cell migration induced by AngII+IL-1β. In agreement, cell migration was induced by 16,16-dimethyl PGE_2 or by the TP agonist U46619 in a concentration dependent manner (Figure 52).

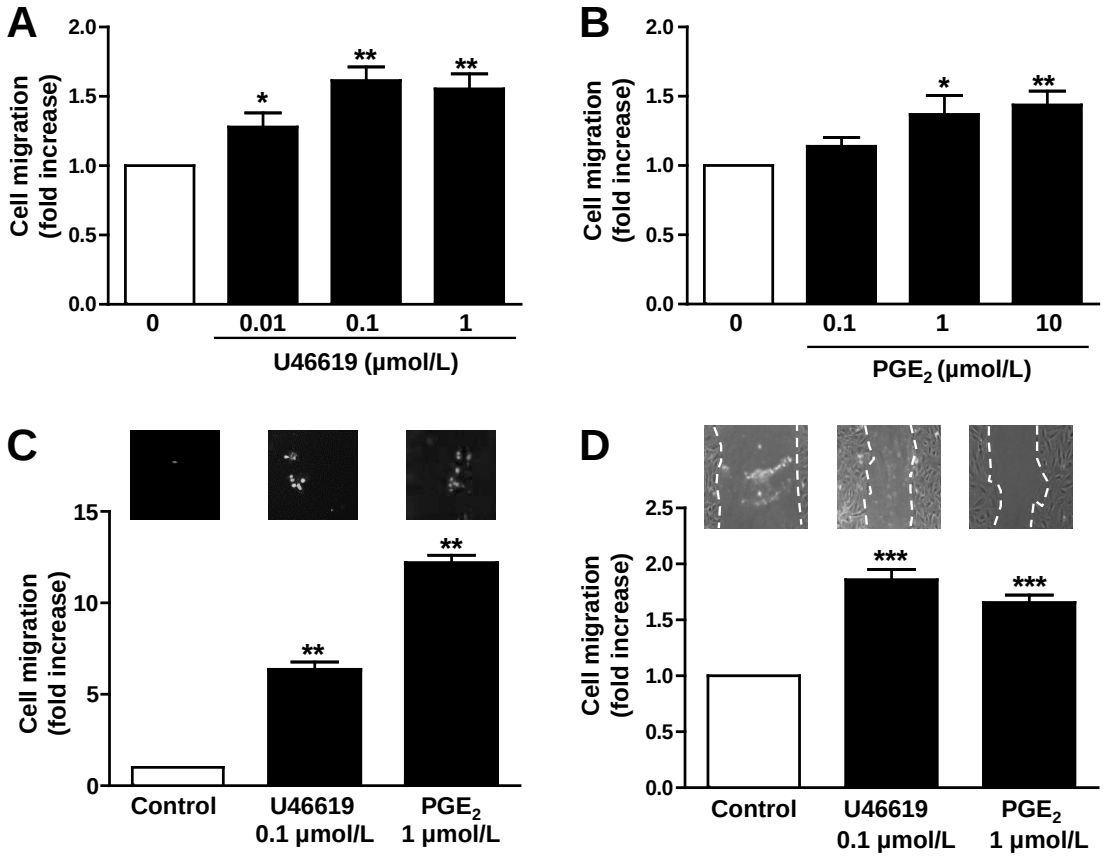

Figure 52. PGE_2 and TXA_2 induce cell migration. *Concentration dependent effect of U46619* ***(A)*** *or PGE2* ***(B)*** *on VSMC migration measured by wound healing. Effects of single dose of U46619 or PGE2 on VSMC migration measured by transwell* ***(C)*** *or would healing* ***(D)****. Data are expressed as mean ± SEM.* $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs Control. n=3-17.*

To understand the signaling whereby TXA_2 and PGE_2 mediate cell migration, we performed wound healing assays in cells stimulated with the TP agonist U46619 (0.1 µmol/L) and 16,16-dimethyl PGE_2 (1 µmol/L) and pretreated with inhibitors of ERK (U0126) JNK (SP600125), p38 MAPK (SB203580), pI3K (LY294002), PKC (chelerythrine, 20 µmol/L), calmodulin (CaM) (W7, 10 µmol/L), pCaMKII (KN93, 20 µmol/L) and EGFR (AG1478, 10 µmol/L); a CaM less potent inhibitor (W12, 10 µmol/L) was also used as a negative control. As shown in Figure 53, only U0126, SP600125, W7, KN93 and AG1478 inhibited 16,16-dimethyl PGE_2- and U46619-induced cell migration indicating the participation of ERK1/2, JNK, CaM, pCaMKII and EGFR in such effects. As expected, cell migration induced by U46619 and 16,16-dimethyl

PGE_2 was also abolished by TP (SQ29548) and EP_1 (SC19220) and EP_3 (L798106) receptor antagonists, respectively (Figure 53).

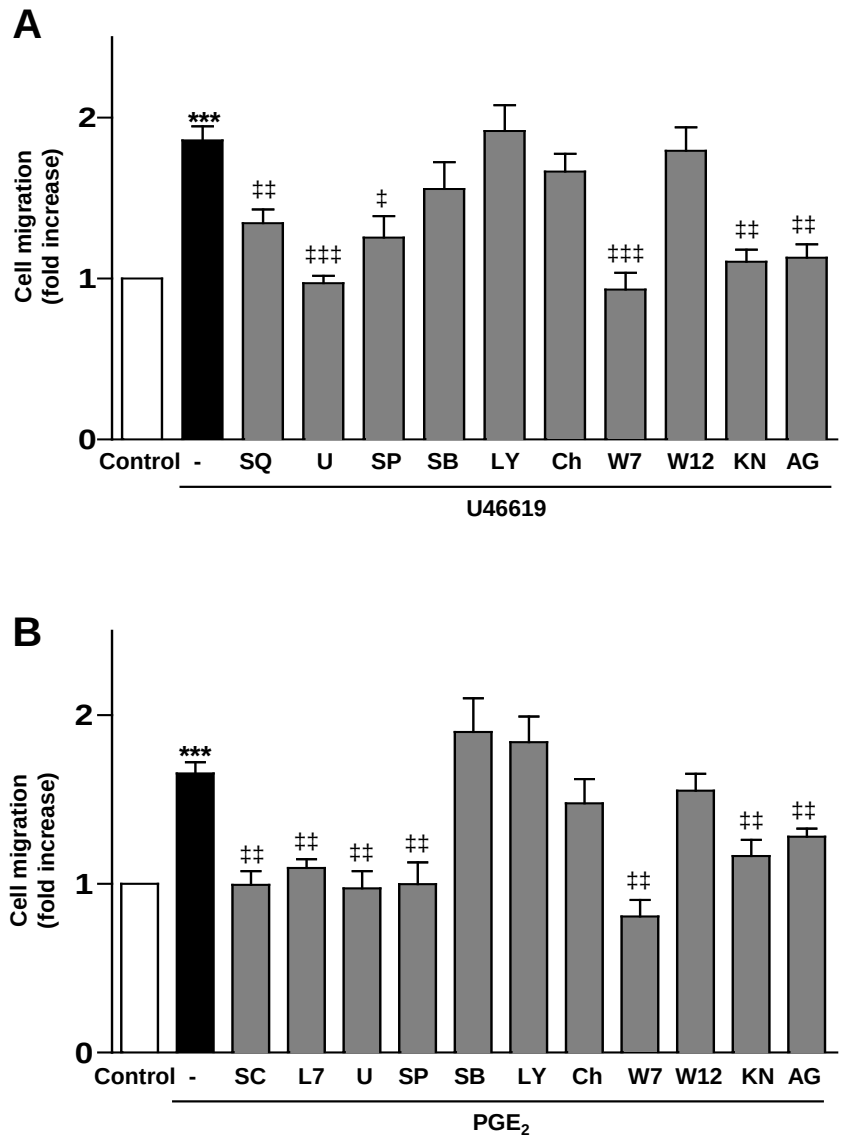

Figure 53. Signaling pathways involved in TXA_2- and PGE_2-dependent cell migration. *Wound healing migration assay in VSMC stimulated with U46619* ***(A)*** *or 16,16-dimethyl PGE_2 (PGE_2)* ***(B)*** *with and without SQ29548 (SQ), SC19220 (SC), L798106 (L7), U0126 (U), SP600125 (SP), SB203580 (SB), LY294002 (LY), chelerythrine (Ch), W7, W12, KN93 (KN) or AG1478 (AG). Data are expressed as mean ± SEM. $^{***}P<0.001$ vs Control; $^{‡}P<0.05$, $^{‡‡}P<0.01$, $^{‡‡‡}P<0.001$ vs U46619 or PGE_2. n=3-6.*

TN-C is expressed predominantly in pathological conditions and it is involved in cell migration (Wang et al., 2011b; Yu et al., 2013). In addition, PGE_2 induces its expression both *in vitro* and *in vivo* (Wang et al., 2011b). As

shown in Figure 54A, TN-C mRNA levels were increased by AngII or IL-1β and this effect was potentiated in the presence of both stimuli. The effect of AngII+IL-1β on TN-C mRNA levels was abolished by celecoxib, U0126, SC19220, L798106 and furegrelate (Figure 54B).

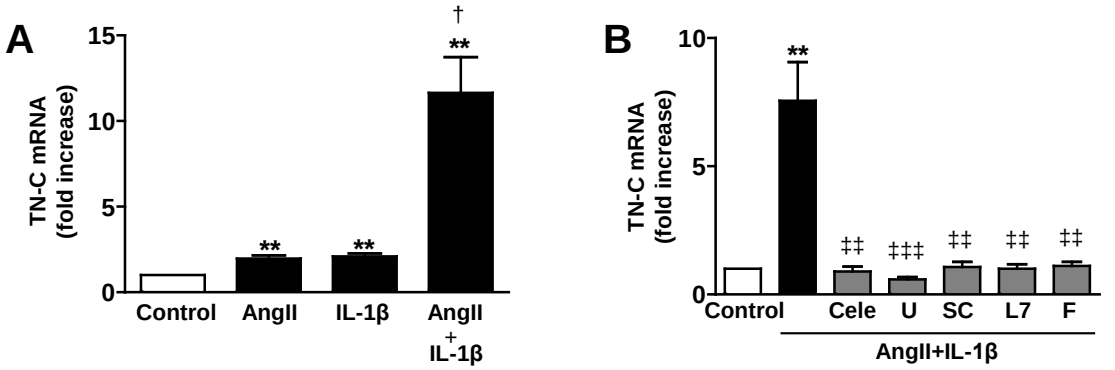

Figure 54. AngII+IL-1β potentiates TN-C expression through COX-2, ERK1/2, PGE_2 and TXA_2 dependent mechanisms. A, *Effect of AngII, IL-1β or AngII+IL-1β (24 h) on TN-C mRNA levels.* ***B,*** *Effect of U0126, celecoxib, SC19220, L798106 or furegrelate on TN-C mRNA levels in VSMC stimulated with AngII+IL-1β (24 h). Data are expressed as mean ± SEM.* $^{**}P<0.01$ *vs Control;* $^{\dagger}P<0.05$ *vs AngII or IL-1β;* $^{‡‡}P<0.01$, $^{‡‡‡}P<0.001$ *vs AngII+IL-1β. n=6.*

All together our results demonstrate that PGE_2 and TXA_2 presumably derived from the ERK1/2/HuR/COX-2 pathway participate of the effects induced by AngII and IL-1β on cell migration by mechanisms involving TN-C and different signaling pathways.

1.5. IMPLICATION OF COX-2-DERIVED PROSTANOIDS IN VASCULAR REMODELING

To study the potential involvement of the results obtained in cultured VSMC, we used two models of vascular remodeling characterized by thickening of the vascular wall influenced by VSMC migration and inflammation.

As shown in Figure 55, increased expression of COX-2, mPGES-1, TXAS and HuR was observed in ligated carotid arteries compared with non-ligated controls.

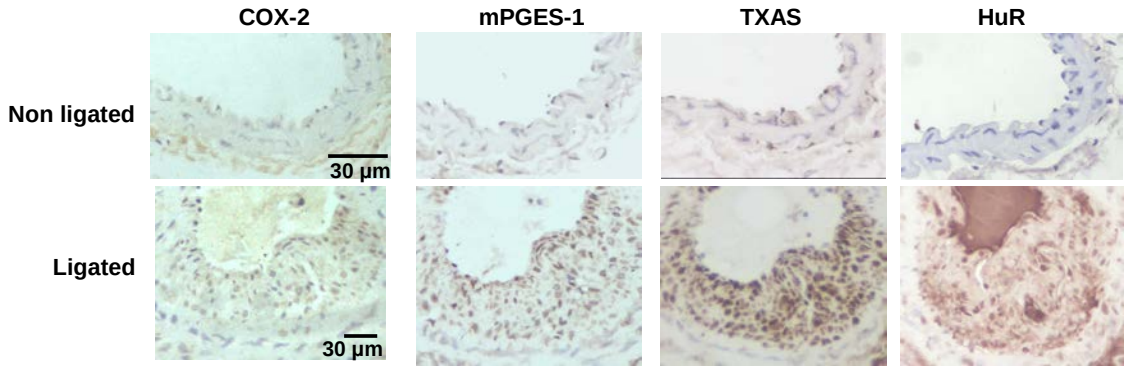

Figure 55. COX-2, mPGES-1, TXAS and HuR expression are up-regulated in carotid ligated arteries. *Representative immunohistochemical staining of COX-2, mPGES-1, TXAS and HuR in non-ligated and ligated carotids. Representative images of 3 independent animals.*

Similar results were obtained in aorta from AngII-infused mice since COX-2, mPGES-1, TXAS and HuR mRNA levels were increased in this model (Figure 56). Moreover, AngII-infused mice showed increased aortic TN-C mRNA levels Figure 56B). Celecoxib treatment did not modify HuR mRNA levels demonstrating that COX-2-derived prostanoids do not affect HuR expression in this model. However, the increased aortic TN-C mRNA levels were reduced by pharmacological COX-2 blockade with celecoxib and by mPGES-1 deletion (Figure 56B).

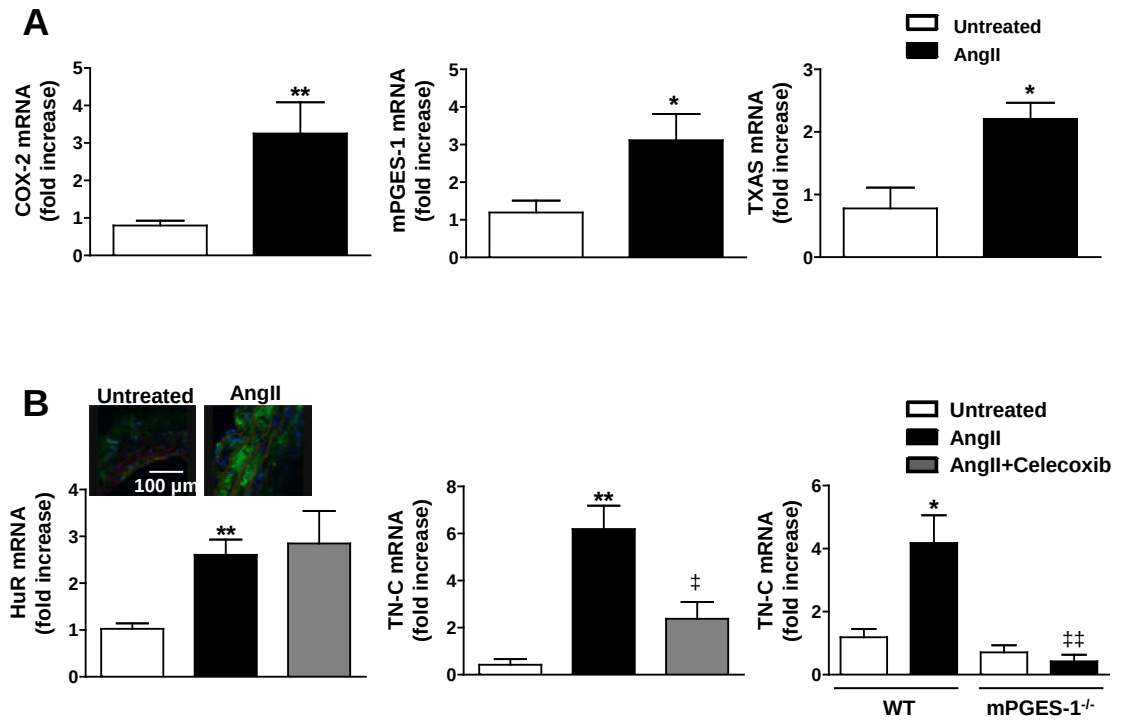

Figure 56. COX-2, mPGES-1, TXAS, HuR and TN-C levels are up-regulated in AngII infused mice. A, *COX-2, mPGES-1 and TXAS mRNA levels in aorta from untreated or AngII-treated mice.* ***B,*** *HuR (mRNA and immunofluorescence) and TN-C mRNA levels in aorta from mice untreated or treated with AngII or AngII plus celecoxib. Aortic TN-C expression in mPGES-1$^{+/+}$ (wild type, WT) and mPGES-1$^{-/-}$ mice infused or not with AngII is also shown. Data are expressed as mean ± SEM. *P<0.05, **P<0.01 vs Untreated; ‡P<0.05, ‡‡P<0.01 vs AngII. n=3-7.*

Aorta from AngII-infused mice also showed increased media:lumen ratio and cross sectional area (markers or hypertrophic remodeling) that was reduced by celecoxib treatment and mPGES-1 deletion (Figure 57).

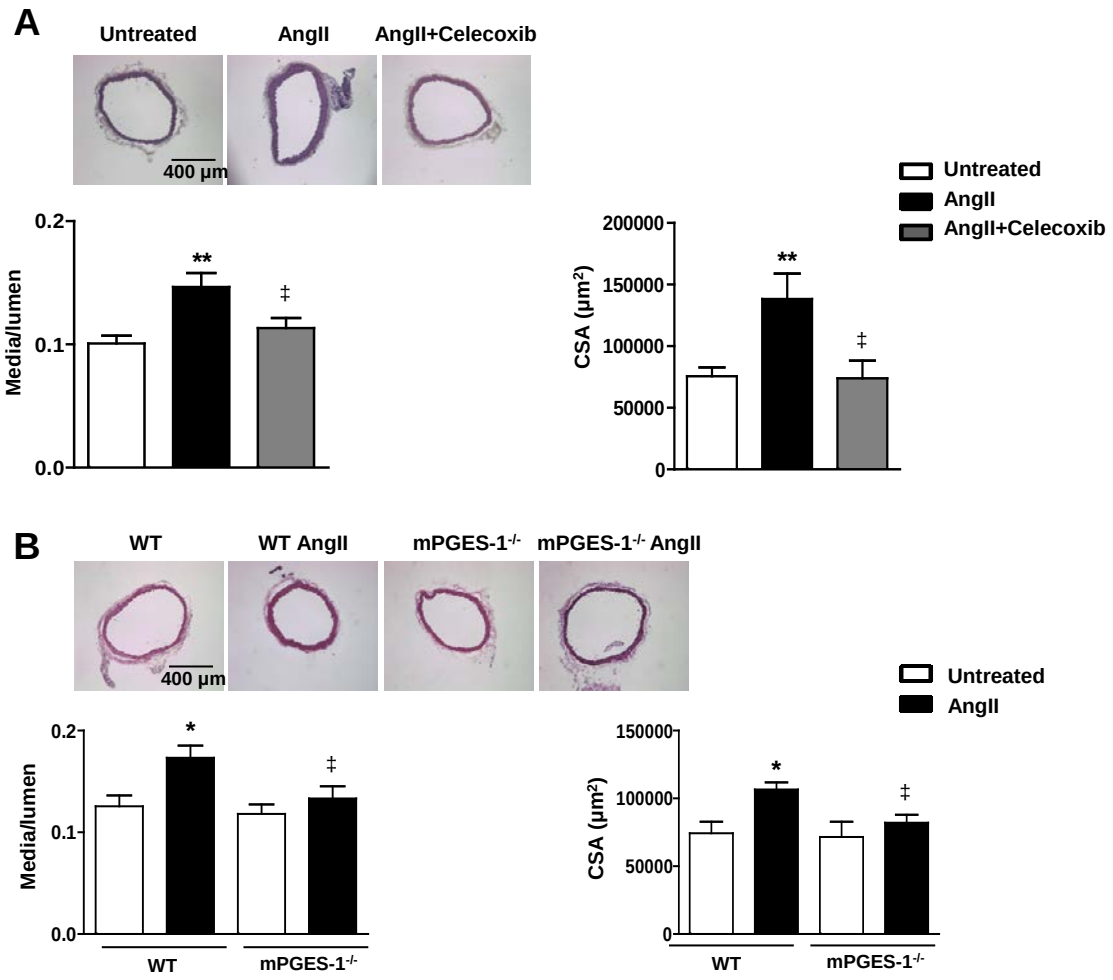

Figure 57. COX-2 and mPGES-1-derived PGE_2 participate in vascular remodeling. *Representative photographs of hematoxylin-eosin aortic sections and media/lumen ratio and cross-sectional-area (CSA) from mice untreated or treated with AngII or AngII plus celecoxib **(A)** and from mPGES-$1^{+/+}$ (wild type, WT) and mPGES-$1^{-/-}$ mice infused or not with AngII **(B)**. Data are expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ vs Untreated; $^{‡}P<0.05$ vs AngII. n=3-7.*

All together these results suggest that the increased expression of COX-2 and mPGES-1 could participate in vascular damage/remodeling by modulating TN-C expression.

2. HuR IS REQUIRED FOR NOX-1 BUT NOT NOX-4 REGULATION BY INFLAMMATORY STIMULI IN VASCULAR SMOOTH MUSCLE CELLS

2.1. ANGII AND IL-1β SYNERGISTICALLY INDUCE NOX-1 EXPRESSION AND DECREASE NOX-4 EXPRESSION IN VSMC

Stimulation of the cells with AngII (0.1 µmol/L) or IL-1β (10 ng/mL) led to a modest increase in NOX-1 mRNA and protein expression (Figure 58). Co-stimulation of cells with AngII+IL-1β led to a synergistic increase in NOX-1 mRNA and protein that become evident after 8 h stimulation and persisted out to 24 h (Figure 58).

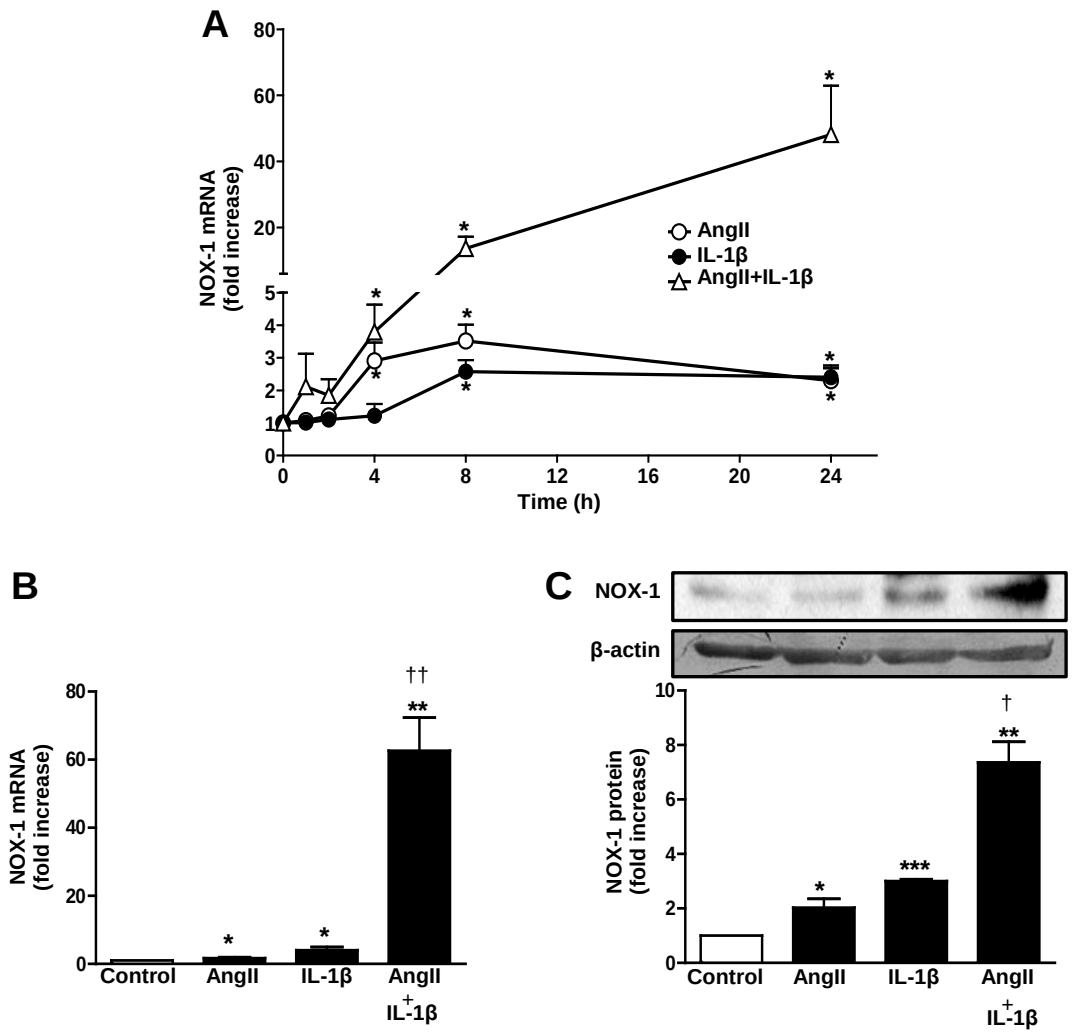

Figure 58. AngII+IL-1β potentiates NOX-1 expression in VSMC. (A) *Time course of NOX-1 mRNA levels in rat VSMC stimulated with AngII, IL-1β or AngII+IL-1β. Effect of AngII, IL-1β or AngII+IL-1β (24 h) on NOX-1 mRNA levels* ***(B)*** *and protein expression* ***(C)****. Representative blots are also shown. Data are expressed as mean ± SEM.* $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs Control;* $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ *vs AngII or IL-1β. n=4-13.*

In contrast, AngII modestly reduced NOX-4 mRNA levels without modifying protein expression and IL-1β robustly decreased NOX-4 mRNA and protein levels (Figure 59). The combination of AngII+IL-1β did not produce further decrease in NOX-4 expression at any time analyzed (Figure 59).

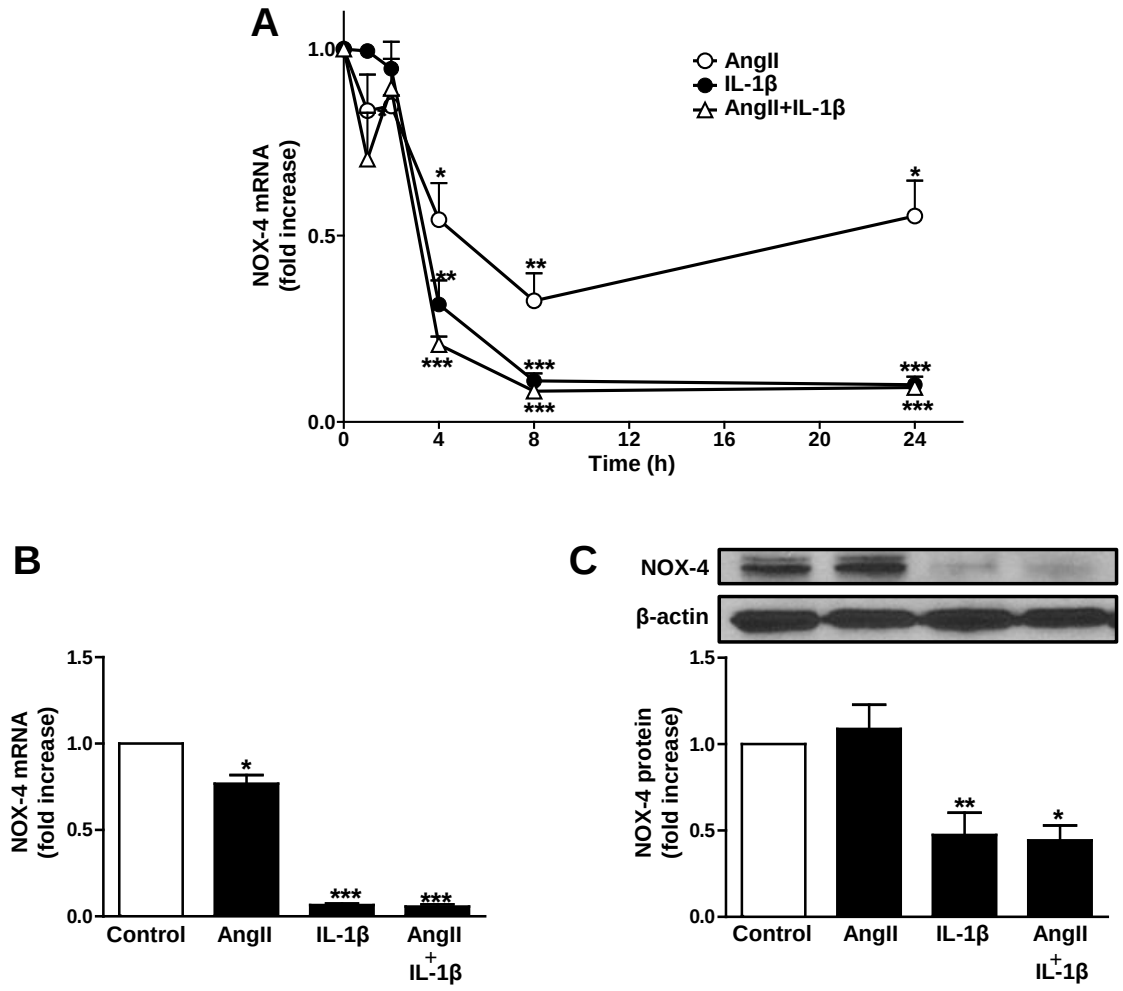

Figure 59. AngII and IL-1β decrease NOX-4 expression in VSMC. ***(A)*** *Time course of NOX-4 mRNA levels in rat VSMC stimulated with AngII, IL-1β or AngII+IL-1β. Effect of AngII, IL-1β or AngII+IL-1β (24 h) on NOX-4 mRNA levels* ***(B)*** *and protein expression* ***(C)****. Representative blots are also shown. Data are expressed as mean ± SEM.* $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs Control. n=4-13.*

In human VSMC stimulated for 24 h, the effects of AngII, IL-1β or AngII+IL-1β on NOX-1 and NOX-4 mRNA levels were similar (Figure 60) indicating an interspecies conserved effect.

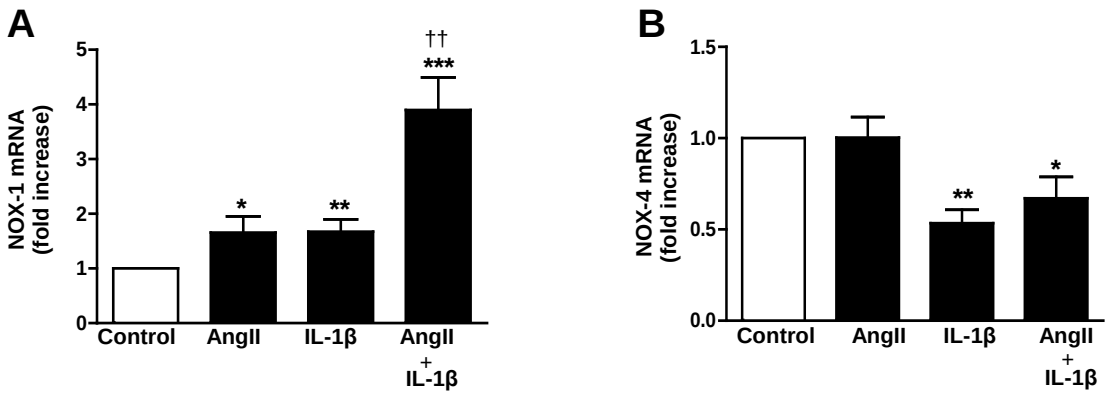

Figure 60. AngII+IL-1β potentiates NOX-1 expression and decreases NOX-4 expression in human VSMC. *Effect of AngII, IL-1β or AngII+IL-1β (24 h) on NOX-1* ***(A)*** *or NOX-4* ***(B)*** *mRNA levels in human VSMC. Data are expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ vs Control; $^{††}P<0.01$ vs AngII or IL-1β. n=7-10.*

Based on these findings, we aimed to analyze the molecular mechanisms responsible for the synergistic effect of AngII+IL-1β on NOX-1 expression and the IL-1β-induced NOX-4 down-regulation in rat VSMC. Pre-treatment with specific inhibitors of ERK1/2 (U0126), JNK (SP600125) and p38 MAPK (SB203580) but not with a PI3K inhibitor (LY294002) reduced the AngII+IL-1β-induced NOX-1 mRNA levels at 24 h (Figure 61A). On the other hand, U0126, SP600125 and LY294002 but not SB203580 partially blocked the IL-1β-dependent NOX-4 down-regulation (Figure 61B).

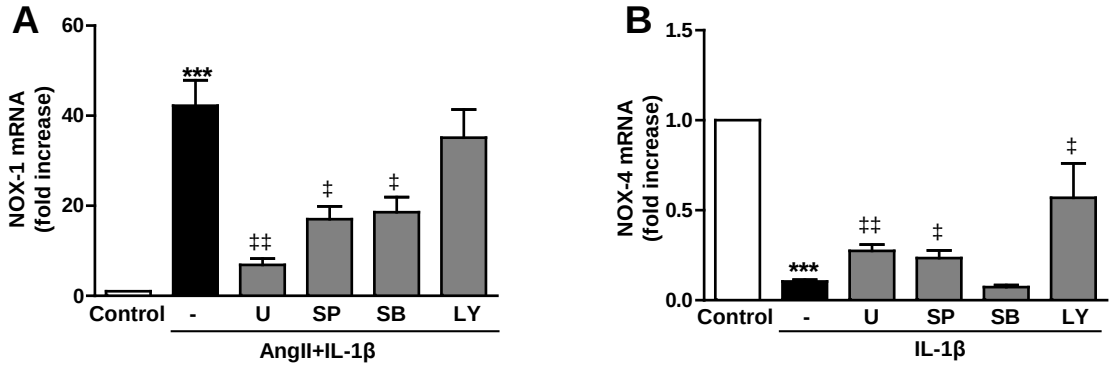

Figure 61. Signaling pathways involved in the effects of AngII and/or IL-1β on NOX-1 and NOX-4 expression. *Effect of inhibitors of ERK1/2 (U, U0126), JNK (SP, SP600125) and p38 MAPK (SB, SB203580) and PI3K (LY, LY294002) on NOX-1* ***(A)*** *and NOX-4* ***(B)*** *mRNA levels induced by AngII and/or IL-1β (24 h). $^{***}P<0.01$ vs Control; $^{‡}P<0.05$, $^{‡‡}P<0.01$ vs AngII+IL-1β or IL-1β. n=7-11.*

2.2. ANGII INDUCES HuR-DEPENDENT NOX-1 mRNA STABILIZATION

Previous studies in VSMC demonstrate that NOX-1 expression is regulated by transcriptional mechanisms (Katsuyama et al., 2005; Cevik et al., 2008; Manea et al., 2010a; 2010b). However, there is a paucity of information on whether post-transcriptional mechanisms are also involved. NOX-1 transcriptional activity was measured in VSMC transfected with a reporter plasmid containing the human NOX-1 promoter fused to luciferase cDNA. As shown in Figure 62, NOX-1 promoter activity increased in the presence of AngII and IL-1β being higher in the presence of both stimuli (Figure 62), confirming that transcriptional regulatory mechanisms are involved in NOX-1 expression induced by AngII+IL-1β.

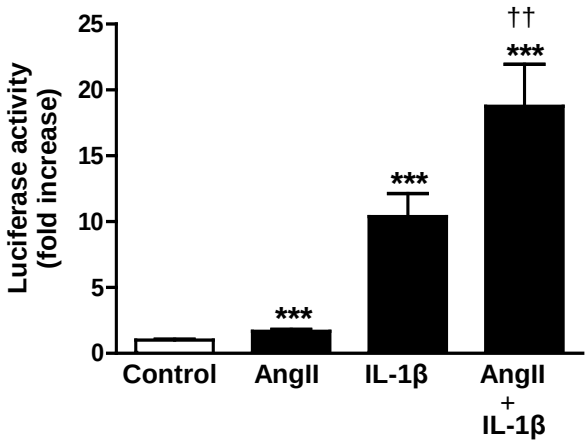

Figure 62. AngII+IL-1β potentiates NOX-1 promoter activity in VSMC. *Luciferase activity of NOX-1 promoter in rat VSMC stimulated with AngII, IL-1β or AngII+IL-1β (24 h). Data are expressed as mean ± SEM. ***P<0.001 vs Control; ††P<0.01 vs AngII or IL-1β. n=6.*

A time-course mRNA decay analysis evidenced that NOX-1 mRNA was stabilized in the presence of AngII+Il-1β (Figure 63A). We also performed transfections of VSMC using a luciferase reporter plasmid containing the 3'UTR from NOX-1 mRNA. Figure 63B shows that AngII+Il-1β increased NOX-1 3'UTR luciferase activity. Together, these results suggest that post-transcriptional regulatory mechanisms are also involved in NOX-1 expression induced by AngII+IL-1β.

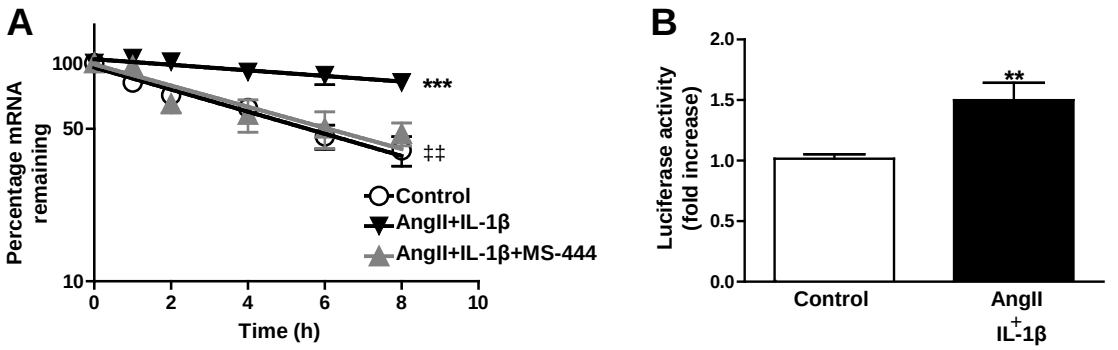

Figure 63. The AngII+IL-1β-induced NOX-1 expression in VSMC is due to an increase of NOX-1 mRNA stability mediated by HuR*. **A,** Effect of MS-444 on NOX-1 mRNA stability in cells stimulated with AngII+IL-1β (24 h). Actinomycin D was added for the indicated times and NOX-1 mRNA levels were measured by qPCR. **B,** Rat VSMC were transfected with a luciferase reporter construct containing the NOX-1 3'-UTR. Luciferase activity was assayed in untreated (control) VSMC and after incubation with AngII+IL-1β (24 h). Data are expressed as mean ± SEM. **P<0.01, ***P<0.001 vs Control; ‡‡P<0.01 vs AngII+IL-1β. n=4-7.*

As mentioned, HuR is a RNA-binding protein that stabilizes different mRNAs by binding to AREs found in 3'UTR (Meisner and Filipowicz, 2011). To test whether HuR was responsible for NOX-1 mRNA stabilization, we performed the mRNA stability assay in the presence of the HuR inhibitor MS-444. As shown in Figure 63A, MS-444 abolished the AngII+IL-1β-dependent NOX-1 mRNA stabilization. Furthermore, HuR blockade with MS-444 (Figure 64A) or with HuR siRNA (Figures 64B and 41A) reduced the AngII+IL-1β-induced NOX-1 mRNA levels.

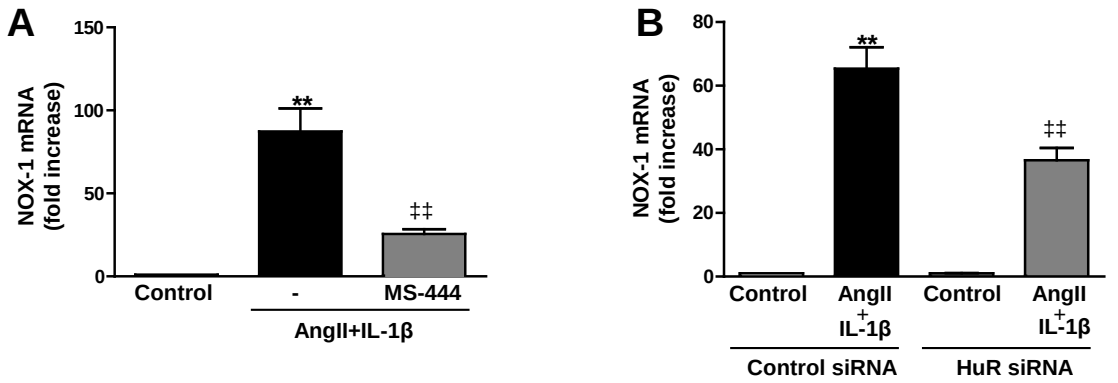

Figure 64. HuR blockade decreases NOX-1 expression induced by AngII+IL-1β. *Effects of MS-444 **(A)** and HuR siRNA **(B)** on NOX-1 mRNA levels in VSMC stimulated with AngII+IL-1β (24 h). Data are expressed as mean ± SEM. **P<0.01 vs Control; ‡‡P<0.01 vs AngII+IL-1β. n=4-7.*

To determine whether AngII+IL-1β signaling promotes HuR binding to NOX-1 mRNA, ribonucleoprotein immunoprecipitation assays were done. VSMC were stimulated for 24 h with AngII+IL-1β and immunoprecipitation of cytoplasmic lysates was performed using an antibody against HuR or a control IgG. We observed enrichment in NOX-1 mRNA in anti-HuR precipitates from cells treated with AngII+IL-1β but not in control cells or in anti-IgG precipitates (Figure 65).

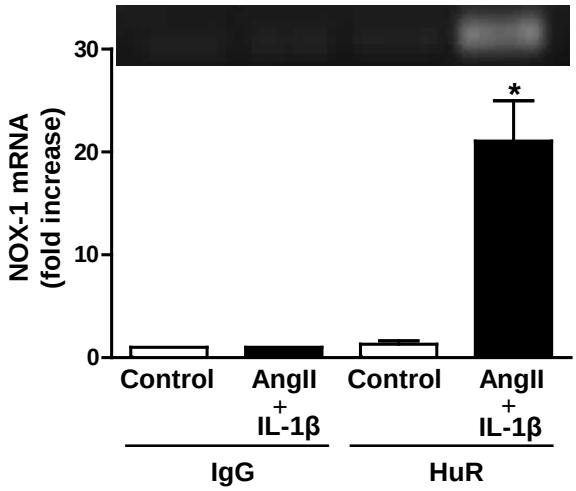

Figure 65. HuR binds to NOX-1 mRNA. *NOX-1 mRNA binding to HuR by ribonucleoprotein immunoprecipitation in VSMC stimulated with AngII+IL-1β (24 h). Data are expressed as mean ± SEM.* **P<0.05 vs Control. n=4.*

Altogether, these findings suggest that AngII+IL-1β induce HuR binding to NOX-1 mRNA which is responsible for increased NOX-1 mRNA stability.

2.3. A TRANSCRIPTIONAL MECHANISM IS INVOLVED IN NOX-4 DOWN-REGULATION INDUCED BY IL-1β

In order to elucidate the mechanisms by which IL-1β decreased NOX-4 expression, we first studied whether a post-transcriptional mechanism was involved by performing a NOX-4 mRNA stability assay. In VSMC, IL-1β did not modify NOX-4 mRNA decay (Figure 66A) supporting the involvement of a transcriptional mechanism. Accordingly, transient transfection assays with a luciferase reporter construct containing NOX-4 promoter, evidenced that IL-1β reduced NOX-4 transcriptional activity (Figure 66B). Interestingly, the decrease in NOX-4 promoter activity triggered by IL-1β was partially reversed with U0126,

SP600125 and LY294002 (Figure 66B) which were the same inhibitors that partially restored the IL-1β-mediated NOX-4 mRNA decrease (Figure 61B).

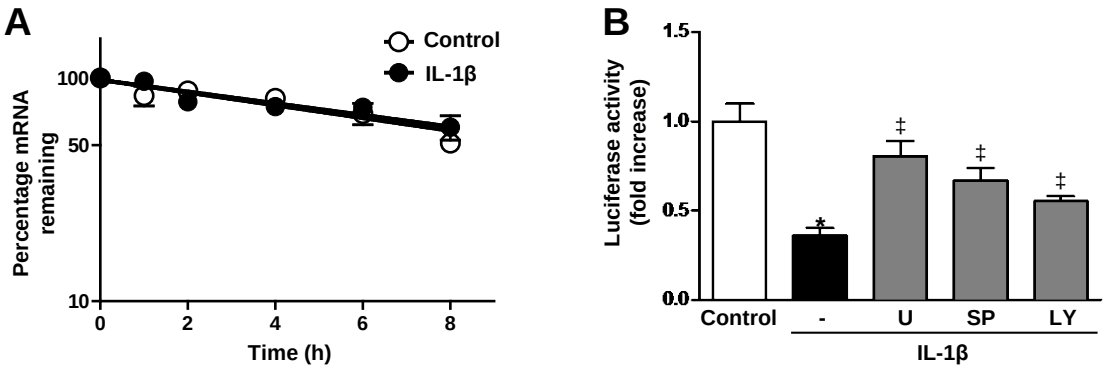

Figure 66. A transcriptional mechanism is involved in NOX-4 down-regulation induced by IL-1β in VSMC. A, *NOX-4 mRNA stability after 24 h stimulation with IL-1β. NOX-4 mRNA levels were measured by qPCR at indicated times after actinomycin D addition.* ***B,*** *Effect of MAPKs (U: U0126; SP: SP600125) and PI3K (LY: LY294002) inhibitors on luciferase activity of NOX-4 promoter in cells incubated with IL-1β (24 h). Data are expressed as mean ± SEM. *P<0.05 vs Control; †P<0.05 vs IL-1β. n=5-12.*

To study which region of the promoter was involved in the NOX-4 transcriptional down-regulation, cells were transfected with different promoter constructs ranging from ~1,200 to 200 bp and stimulated with IL-1β. Luciferase activities of all constructs were decreased in IL-1β-treated cells, suggesting that the sequence responsible for NOX-4 down-regulation was within the proximal 192 bp of NOX-4 promoter (Figure 67).

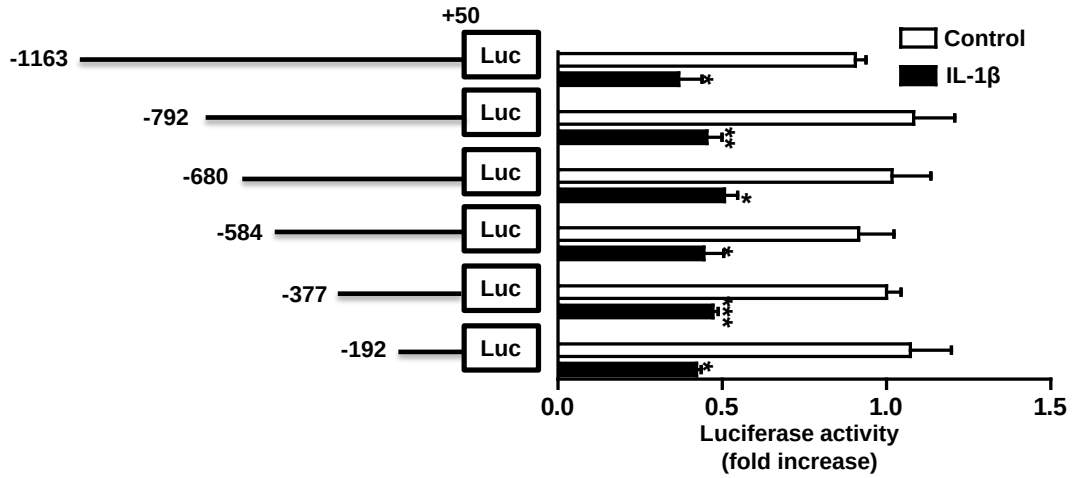

Figure 67. NOX-4 promoter of 192 bp is involved in NOX-4 down-regulation induced by IL-1β in VSMC. *Luciferase activity of rat VSMC transfected with luciferase reporter constructs containing different lengths of the human NOX-4 promoter and stimulated with IL-1β (24 h). Scheme of the constructs is also shown. Data are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs Control. n=5-12.*

Epigenetic modifications are mechanisms involved in gene expression. These mechanisms are reversible and involve DNA methylation and histone deacetylation leading to a major compaction of the chromatin and thus inhibition of gene expression. Very few is known about epigenetic modifications and NOX-4 expression; for example in cancer cells, NOX-4 expression is regulated by DNA methylation (De Carvalho et al., 2012). We used the inhibitors of DNA methyltransferase 5-aza-dC, and of HDAC trichostatin and SAHA, to determine whether epigenetic modifications were involved in the observed NOX-4 transcriptional repression induced by IL-1β. As shown in Figure 68, 5-aza-dC (2 μmol/L) did not affect basal or IL-1β-mediated NOX-4 mRNA decrease; however, trichostatin (0.5 μmol/L) or SAHA (5 μmol/L) greatly decreased basal NOX-4 mRNA levels and no further effect was observed with IL-1β, probably because no further diminution can be achieved.

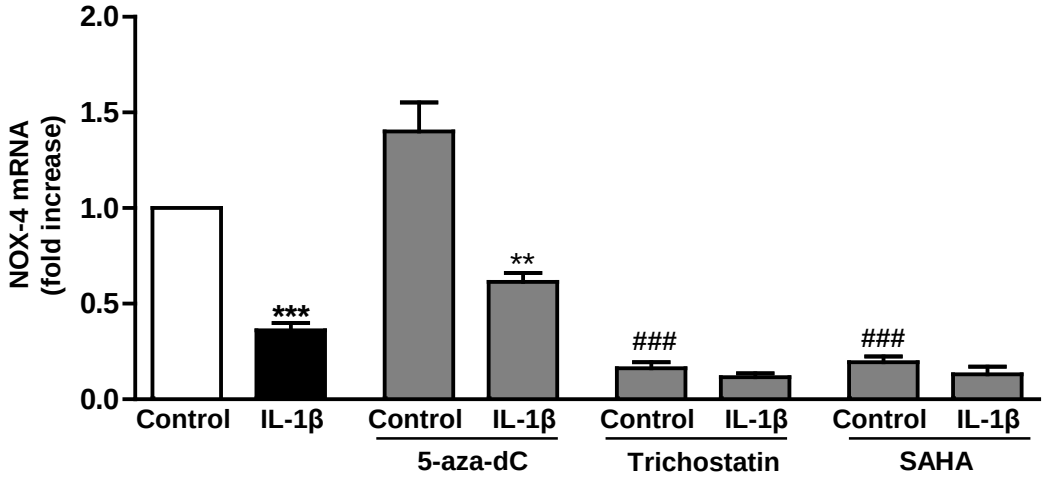

Figure 68. HDAC participates in NOX-4 basal expression. *Effects of 5-aza-2'-deoxycytidine (5-aza-dC), trichostatin and suberoylanilide hydroxamic acid (SAHA) on NOX-4 mRNA levels in VSMC unstimulated (control) or stimulated with IL-1β (8 h). Data are expressed as mean ± SEM. $^{**}P<0.01$; $^{***}P<0.001$ vs Control; $^{###}P<0.001$ vs Control in the absence of inhibitors. n=5-12.*

In VSMC transfected with the 192 bp NOX-4 promoter, trichostatin and SAHA greatly repressed NOX-4 basal transcriptional rate without affecting IL-1β effects (Figure 69). This suggests that deacetylation is needed for the basal expression of NOX-4.

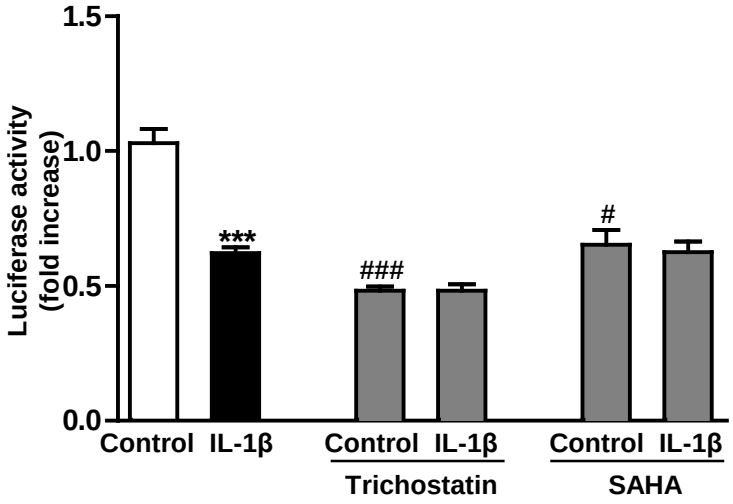

Figure 69. HDAC participates in NOX-4 basal promoter activity. *Effects of trichostatin and suberoylanilide hydroxamic acid (SAHA) on luciferase activity of NOX-4 promoter in VSMC transfected with the NOX-4 192 promoter construct and stimulated or not with IL-1β (8 h). Data are expressed as mean ± SEM. ***P<0.001 vs unstimulated (control); $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs control in the absence of inhibitors. n=5-12.*

To gain further insights into the mechanisms involved in the IL-1β-mediated NOX-4 decrease, VSMC were treated with the protein synthesis inhibitor cycloheximide (25 µg/mL) before stimulation. As shown in Figure 70, cycloheximide abolished the effect of IL-1β on NOX-4 expression suggesting that the new synthesis of a repressor is needed for the IL-1β-induced NOX-4 decrease.

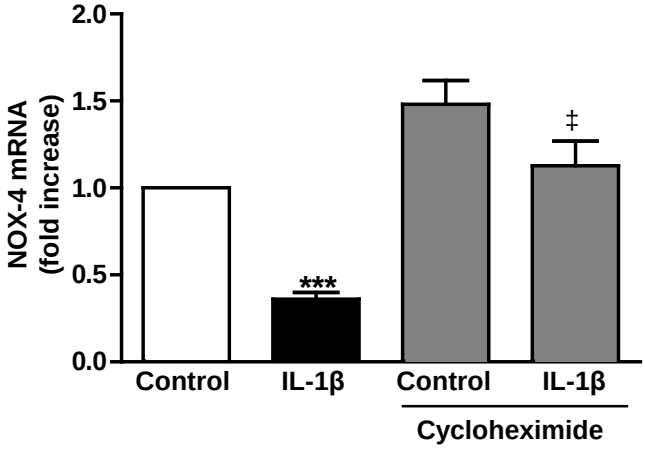

Figure 70. New synthesis of a repressor is required for NOX-4 down-regulation induced by IL-1β in VSMC. *Effect of cycloheximide on NOX-4 mRNA levels in VSMC stimulated with IL-1β (8 h). Data are expressed as mean ± SEM. ***P<0.001 vs Control; †P<0.05 vs IL-1β. n=9-12.*

Thereafter, we studied the expression of some transcription factors that could act as repressors that bind to the 192 bp proximal promoter. Elk-1 and PPAR-γ mRNA levels were unchanged and decreased, respectively, after IL-1β stimulation (Figure 71).

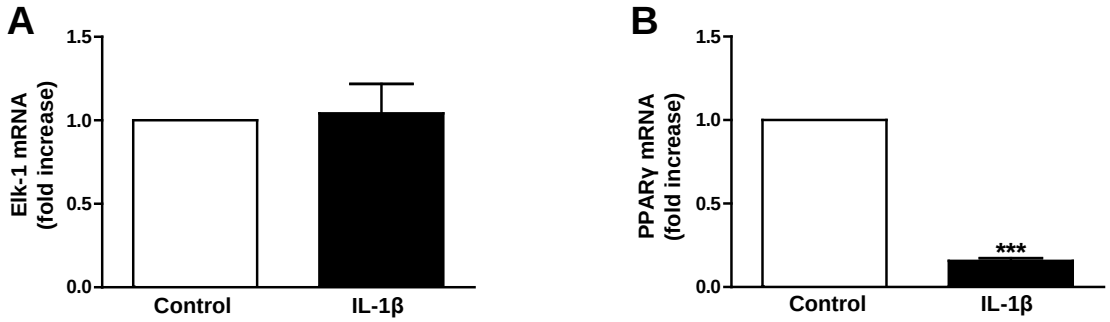

Figure 71. Elk-1 and PPAR-γ transcription factors are unaffected and decreased by IL-1β. *Effects of IL-1β (24 h) on Elk-1* ***(A)*** *and PPAR-γ* ***(B)*** *mRNA levels in rat VSMC. Data are expressed as mean ± SEM.* ****P<0.001 vs Control. n=6-11.*

However, Elf-3 and Egr-1 mRNA levels were increased in the presence of IL-1β (Figure 72A and 72B). The Elf-3 dominant negative mutant did not affect IL-1β-mediated NOX-4 mRNA down-regulation (Figure 72C) and Egr-1 overexpression increased NOX-4 expression (Figure 72D), suggesting no implication of these transcription factors in the IL-1β-dependent NOX-4 decrease.

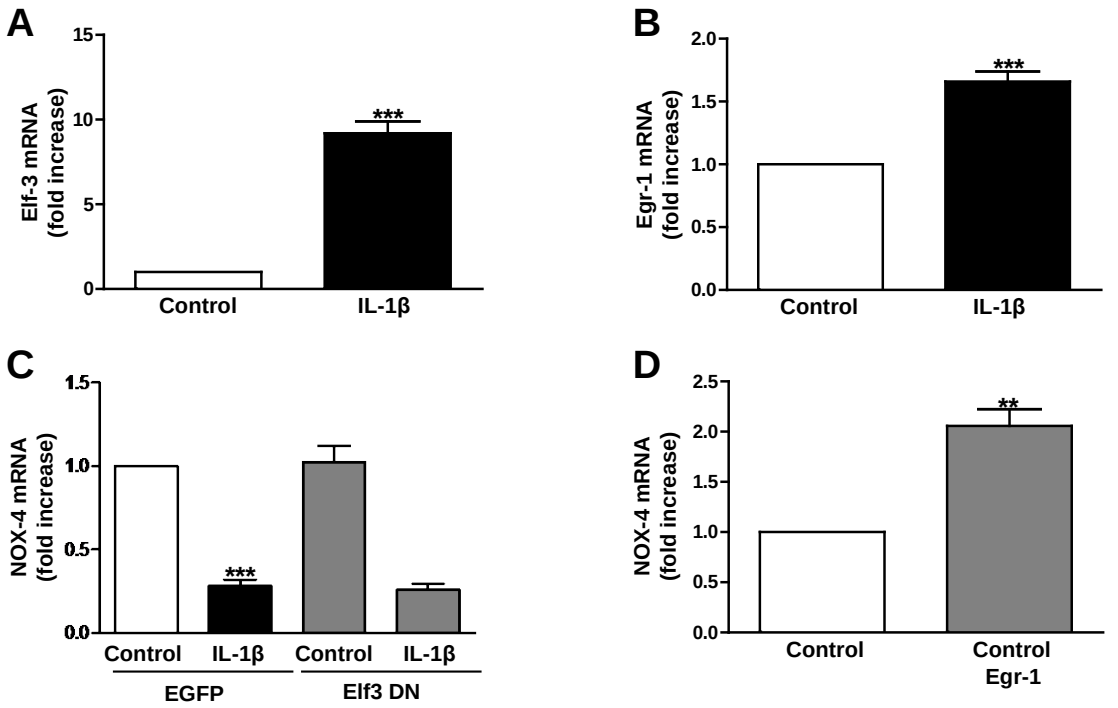

Figure 72. Elf-3 and Egr-1 transcription factors are not involved in IL-1β-mediated NOX4 down-regulation. *Effects of IL-1β (24 h) on Elf-3* ***(A)*** *and Egr-1* ***(B)*** *mRNA levels in rat VSMC.* ***(C)*** *NOX-4 mRNA levels in VSMC transfected with EGFP or Elf-3 dominant negative mutant (Elf-3 DN) and stimulated or not with IL-1β.* ***(D)*** *NOX-4 mRNA levels in VSMC transfected with pcDNA3.1 (Control) or Egr-1. Data are expressed as mean ± SEM.* ***P<0.01,* ****P<0.001 vs Control. n=6-11.*

2.4. NOX-1-DERIVED ROS AND NOX-4-DERIVED H_2O_2 PARTICIPATE IN CELL MIGRATION

We next studied whether the regulatory mechanisms observed in NOX-1 and NOX-4 expression have a functional role on NOXs activity and their presumed effects on cell migration.

NOX-1 has been described to produce $O_2^{\bullet-}$ while H_2O_2 is produced, at least in part, by NOX-4 (Dikalov et al., 2008). AngII or IL-1β induced NADPH oxidase activity and ROS production that were further increased by the combination of both stimuli (Figure 73A and 73B). AngII+IL-1β-induced NADPH oxidase activation and ROS production were blocked by a NOX-1 inhibitor (ML171, 0.5 µmol/L) and by NOX-1 siRNA (Figure 73).

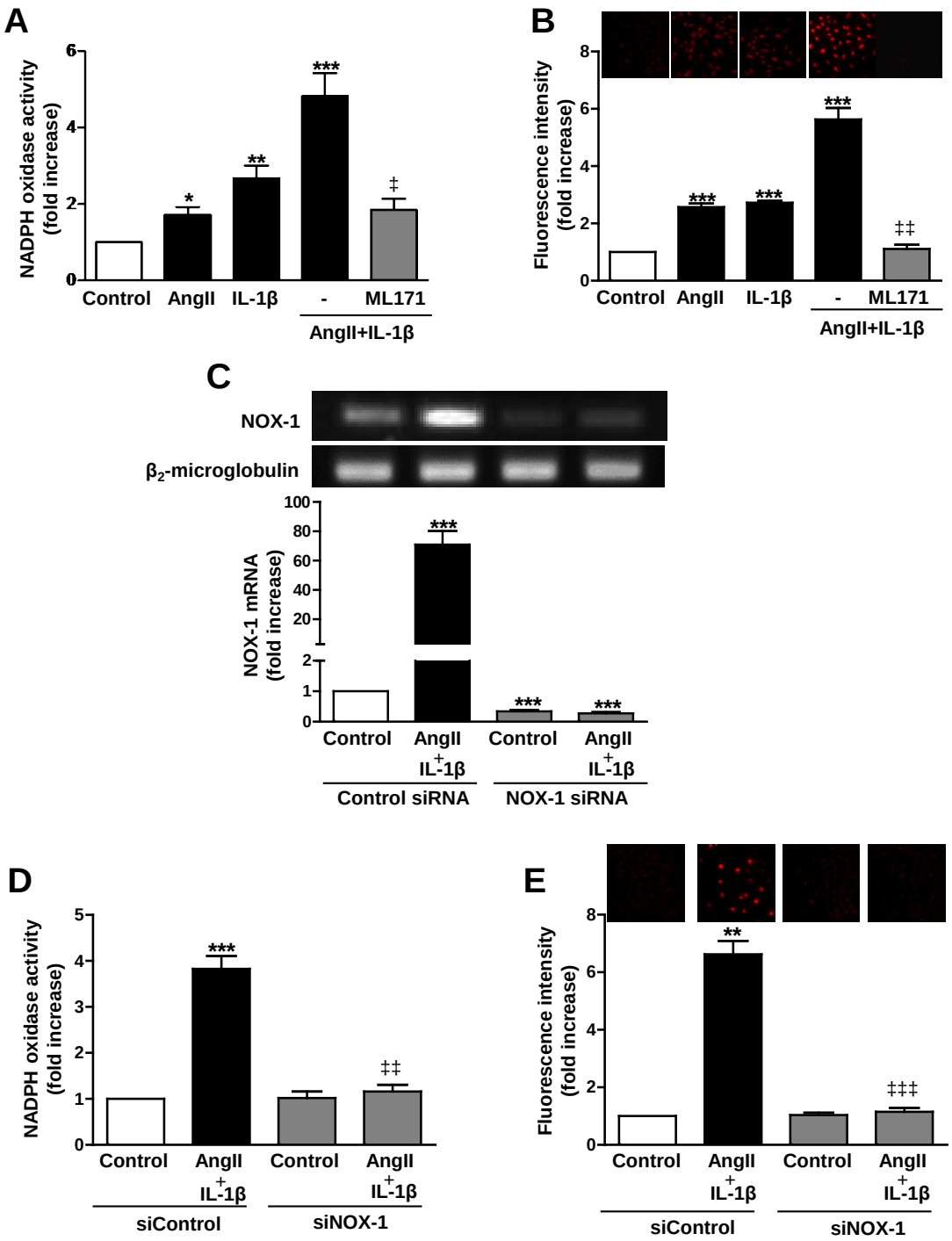

Figure 73. NOX-1 is responsible for AngII+IL-1β-induced ROS production. *Effect of ML171* ***(A,B)*** *and NOX-1 siRNA* ***(C-E)*** *on NADPH oxidase activity and ROS production induced by AngII, IL-1β and AngII+IL-1β in rat VSMC (24 h).* ***C,*** *NOX-1 mRNA levels in cells transfected with control or NOX-1 siRNA stimulated or not with AngII+IL-1β (24 h). Data are expressed as mean ± SEM.* $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs Control;* $^{‡}P<0.05$, $^{‡‡}P<0.01$, $^{‡‡‡}P<0.001$ *vs AngII+IL-1β. n=3-7.*

We have previously demonstrated that HuR is involved in AngII+IL-1β-induced NOX-1 expression. The role of HuR on NOX activity was confirmed by

the fact that HuR knockdown by siRNA partially decreased NADPH oxidase activity and ROS production induced by AngII+IL-1β (Figure 74).

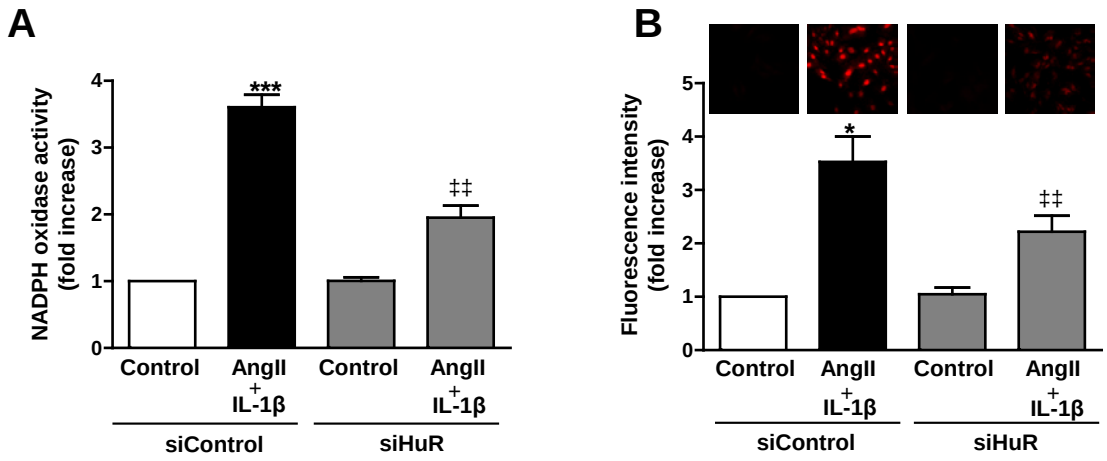

Figure 74. HuR participates in AngII+IL-1β-induced ROS production. *Effects of control or HuR siRNA on NADPH oxidase activity* ***(A)*** *and ROS production* ***(B)*** *in VSMC stimulated with AngII+IL-1β. Data are expressed as mean ± SEM.* **P<0.05,* ****P<0.001 vs Control;* ‡‡*P<0.01 vs AngII+IL-1β. n=3-7.*

We next evaluated whether effects of AngII and/or IL-1β on NOX-4 expression were paralleled by changes in H_2O_2 production. As shown in Figure 75A, H_2O_2 production was decreased in the presence of AngII being this effect more exacerbated in the presence of IL-1β or AngII+IL-1β suggesting that NOX-4 might be the responsible for H_2O_2 production. In agreement, NOX-4 over-expression abolished the decrease induced by IL-1β in H_2O_2 production, (Figure 75B).

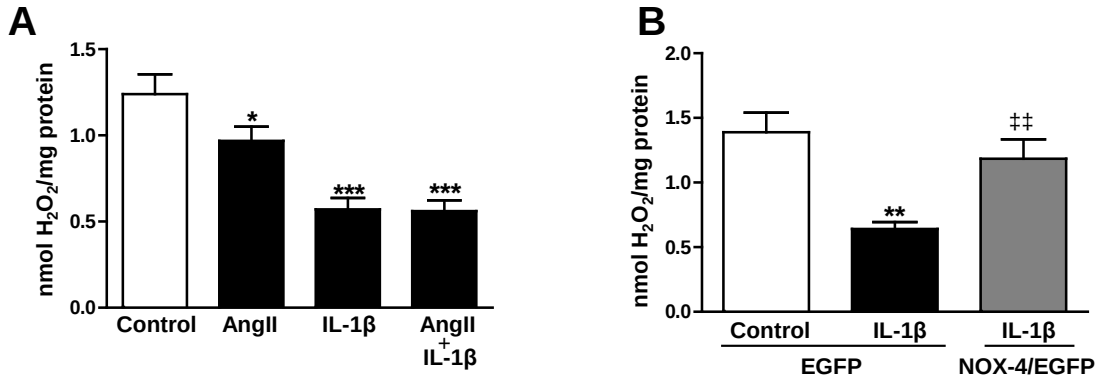

Figure 75. NOX-4 participates in IL-1β-dependent H_2O_2 production. *H_2O_2 production in VSMC stimulated with AngII, IL-1β or AngII+IL-1β* ***(A)*** *or transfected with EGFP or NOX-4/EGFP and stimulated with IL-1β* ***(B)****. Data are expressed as mean ± SEM.* **P<0.05,* ***P<0.01,* ****P<0.001 vs Control;* ‡‡*P<0.01 vs IL-1β. n=3-7.*

Transwell and wound healing assays were used to study the implication of ROS in VSMC migration. We previously observed that 5 h stimulation with AngII and/or IL-1β induced VSMC migration (Figure 49). This was confirmed herein after 24 h stimulation (Figure 76).

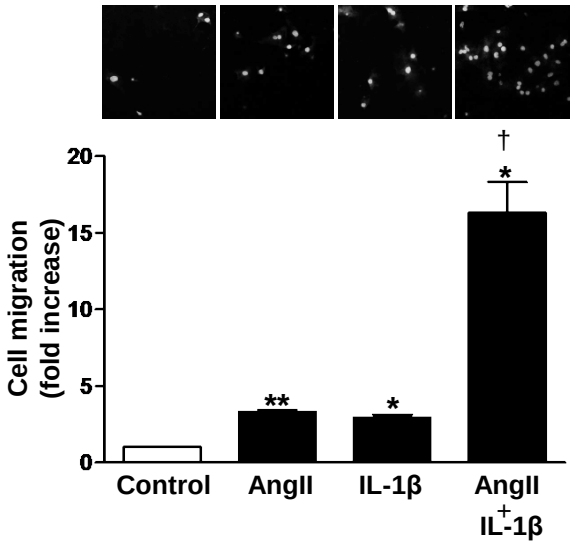

Figure 76. AngII+IL-1β potentiates cell migration. *Transwell assay performed in rat VSMC unstimulated and stimulated with AngII, IL-1β or AngII+IL-1β for 24 h. Data are expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ vs Control; $^{\dagger}P<0.05$ vs AngII or IL-1β. n=3.*

We previously showed that HuR was involved in AngII+IL-1β-induced cell migration (Figure 51). We now compared the effects of NOX-1 and HuR blockade on cell migration induced by AngII+IL-1β. As expected, MS-444 and HuR siRNA inhibited AngII+IL-1β-induced VSMC migration (Figure 77) and these effects were mimicked by ML171 and NOX-1 siRNA (Figure 77) suggesting that NOX-1 which is under the control of HuR, is involved in VSMC migration.

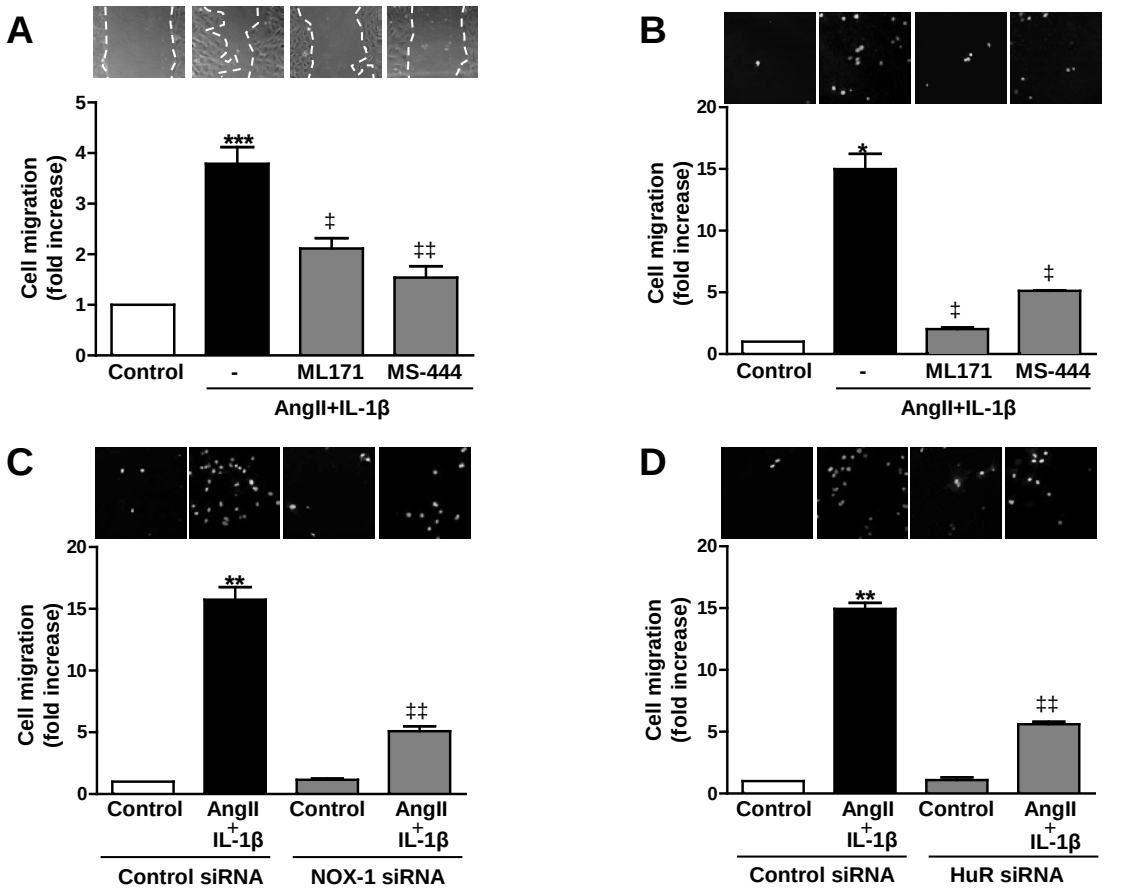

Figure 77. NOX-1 and HuR participate in cell migration induced by AngII+IL-1β. *Effect of ML171 and MS-444 on cell migration induced by AngII+IL-1β measured by wound healing* ***(A)*** *and transwell* ***(B)*** *assays. Effect of NOX-1* ***(C)*** *or HuR* ***(D)*** *siRNAs on cell migration induced by AngII+IL-1β measured by transwell assays. Data are expressed as mean ± SEM.* $^{**}P<0.01$ *vs Control;* $^{‡}P<0.05$, $^{‡‡}P<0.01$ *vs AngII+IL-1β. n=3-5.*

In order to study the participation of NOX-4 in cell migration, we overexpressed NOX-4 in the presence of IL-1β. As shown in Figure 78, overexpression of NOX-4 increased IL-1β-induced cell migration.

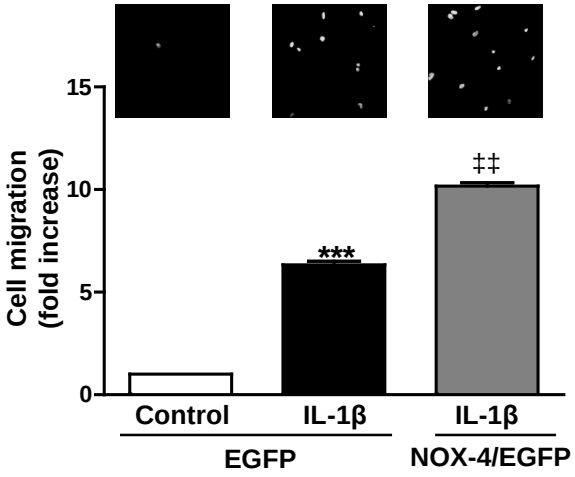

Figure 78. NOX-4 participates in cell migration. *Effect of NOX-4/EGFP over-expression on cell migration induced by IL-1β measured by transwell assays. Data are expressed as mean ± SEM. ***P<0.001 vs Control; ‡‡P<0.01 vs IL-1β. n=3-5.*

Altogether these results suggest the implication of the HuR/NOX-1 axis and NOX-4 on VSMC migration in response to inflammatory stimuli.

2.5. LACK OF RECIPROCAL RELATIONSHIP BETWEEN COX-2 AND NOX-1

A reciprocal relationship between COX-2 and NADPH oxidase has been described in liver cells (Sancho et al., 2011) and arteries from hypertensive animals (Martínez-Revelles et al., 2013). We evaluated whether this reciprocal relationship was part of the mechanisms responsible of the effects of AngII+IL-1β on COX-2 and NOX-1 expression. COX-2 and NOX-1 mRNA levels and NADPH oxidase activity were analyzed in VSMC stimulated with AngII+IL-1β in the presence or absence of ML171 or celecoxib. As shown in Figure 79A, COX-2 expression was not affected by ML171. Conversely, although NOX-1 expression was slightly inhibited by celecoxib (Figure 79B) the NADPH oxidase activity was not modified by this compound (Figure 79C). These results demonstrate that in these particular experimental conditions, COX-2 and NOX-1 pathways seem to be independently activated by AngII+IL-1β.

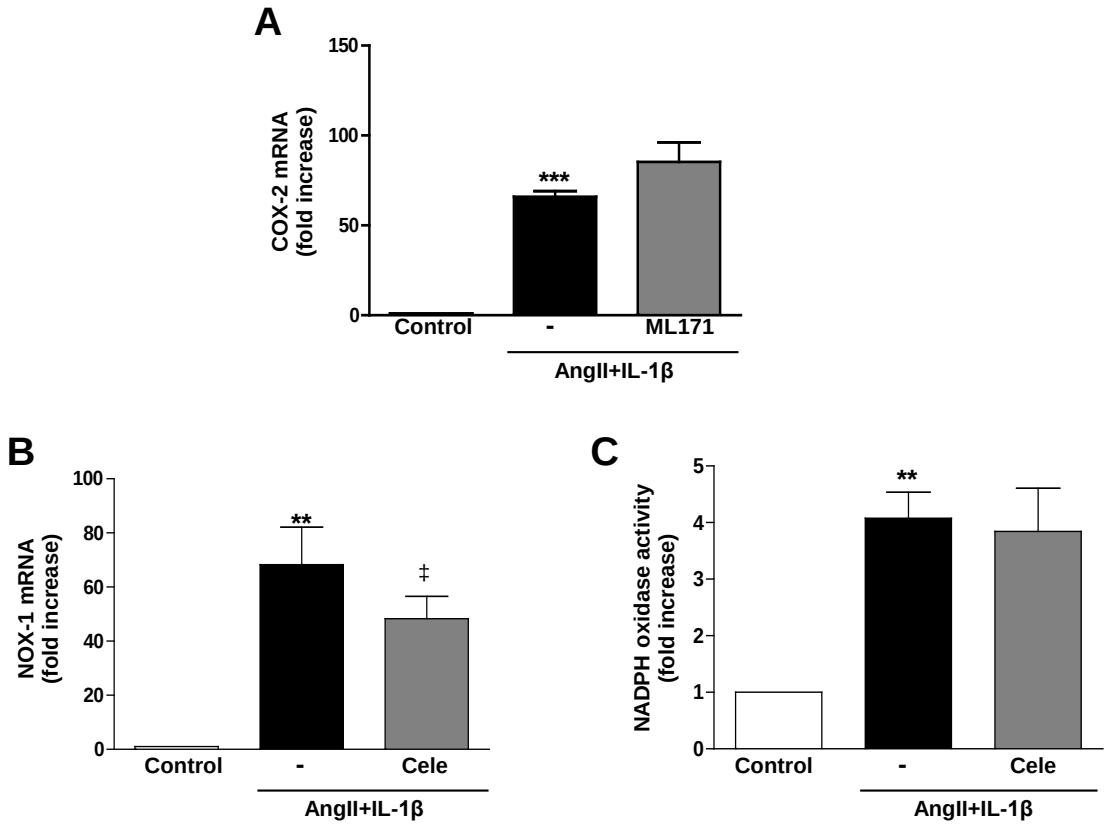

Figure 79. Lack of reciprocal relationship between COX-2 and NOX-1. A*, Effect of ML171 on COX-2 mRNA levels. Effect of celecoxib (Cele) on NOX-1 mRNA levels* ***(B)*** *and NADPH oxidase activity* ***(C)*** *in VSMC stimulated with AngII+IL-1β (24 h). Data are expressed as mean ± SEM.* $^{***}P<0.001$ *vs Control;* $^{‡}P<0.05$ *vs AngII+IL-1β. n=6.*

Discussion

1. HuR MEDIATES THE SYNERGISTIC EFFECTS OF ANGII AND IL-1β ON VASCULAR COX-2 EXPRESSION AND CELL MIGRATION

In this part of the PhD Thesis, we present evidences that the combination of two inflammatory stimuli, AngII and IL-1β, synergistically induce COX-2 expression and cell migration in VSMC via a HuR-mediated increase of mRNA stability. We propose that this mechanism might be implicated in the vascular remodeling associated to cardiovascular diseases.

It is well known that AngII and cytokines have been implicated in several cardiovascular pathologies through their inflammatory actions in the vascular wall (Marchesi et al., 2008). COX-2 is an early response gene overexpressed in pathological conditions associated with inflammatory processes, such as cancer (Dixon et al., 2001; Young et al., 2012), atherosclerosis (Cipollone and Fazia, 2006) and hypertension (Martínez-Revelles et al., 2013; Virdis et al., 2013). We found that AngII and IL-1β time-dependently increased COX-2 protein levels in VSMC, as described in this and other cell types (Ohnaka et al., 2000; Slice et al., 2005; Martín et al., 2012). However, the temporal pattern of COX-2 up-regulation by these stimuli was different. IL-1β induced a sustained long-term COX-2 up-regulation, whereas AngII induced an earlier response that was attenuated after prolonged stimulation times. Importantly, the combination of AngII+IL-1β leads to an exacerbated and more sustained COX-2 induction suggesting that amplifying mechanisms of COX-2 regulation might take place to increase the inflammatory response. In agreement, in intestinal epithelial cells (Pham et al., 2008), mesangial cells (Doller et al., 2008) or adventitial fibroblasts (Galán et al., 2011), AngII potentiated COX-2 expression induced by growth factors or pro-inflammatory cytokines. One of the main consequences of vascular inflammation relates to the effects of pro-inflammatory mediators in vascular structure. In fact, COX-2-derived prostanoids are known to promote VSMC migration as demonstrated by both *in vitro* and *in vitro* studies (Wang et al., 2005; Zhang et al., 2013). In agreement, we observed that the effects of AngII+IL-1β on COX-2 expression in VSMC had a functional consequence since the combination of both stimuli synergistically induced VSMC migration by a mechanism dependent on COX-2-derived prostanoids.

COX-2 expression is tightly regulated by both transcriptional and post-transcriptional mechanisms (Dixon et al., 2000; Harper and Tyson-Capper, 2008; Young et al., 2012). Pham et al., (2008) described that the synergistic up-regulation of COX-2 induced by AngII and epidermal growth factor in intestinal epithelial cells was due to an increase of its transcriptional rate and its mRNA stability. Our results in VSMC demonstrate that the effect of AngII on IL-1β-induced COX-2 expression was mainly due to a mRNA stabilization mechanism, as previously described in adventitial fibroblasts (Galán et al., 2011). RNA stability is regulated by different mRNA binding proteins such as HuR, which is most noted to play a role in cancer through stabilization of various target genes such as COX-2 (Dixon et al., 2001; Abdelmohsen and Gorospe, 2010). HuR is mainly located in the nucleus and shuttles between the nucleus and the cytoplasm. The presence of cytoplasmic HuR contribute to promote cell growth, proliferation, angiogenesis and survival (Abdelmohsen and Gorospe, 2010), through its capacity to stabilize ARE-containing mRNAs such as cyclins, TNF-α, vascular endothelial growth factor, and COX-2 (Abdelmohsen and Gorospe, 2010). This is evident in colorectal and other tumor types, where HuR is overexpressed and present within the cytoplasm leading to ARE-mRNA stabilization and increased of COX-2 expression (Dixon et al., 2001; Young et al., 2012). Post-translational modifications of HuR including serine and threonine phosphorylation by several kinases such as Chk2, PKCα or PKCδ can modify HuR subcellular location (Meisner and Filipowicz, 2011). Additionally, ERK1/2 can participate in HuR phosphorylation, modifying its activity as described in a lung cancer cell line (Yang et al., 2004b) or modifying HuR cytoplasmic location as shown in hepatic stellate cells (Woodhoo et al., 2012). Herein, we demonstrate for the first time in VSMC that ERK1/2-mediated HuR activation is involved in the synergistic effect of AngII and IL-1β on COX-2 expression and cell migration. This is supported by the following findings: 1) AngII+IL-1β interfered with rapid decay of COX-2 mRNA resulting in increased 3'UTR reporter activity in VSMC. This effect was accompanied with HuR translocation to the cytoplasm and its binding to COX-2 mRNA; 2) HuR silencing and/or treatment with the HuR inhibitor MS-444 reduced COX-2 mRNA stability, COX-2 mRNA levels and cell migration induced by AngII+IL-1β; 3) ERK1/2 phosphorylation was increased by AngII+IL-1β and

ERK1/2 inhibition reduced COX-2 mRNA stability, COX-2 mRNA levels, HuR cytoplasmic translocation and cell migration. All together, these results suggest that HuR cytoplasmic trafficking could be a central node implicated in vascular remodeling associated to cardiovascular pathologies by modulating COX-2 expression and cell migration. In agreement, we observed increased vascular expression of HuR in AngII-infused mice and in the carotid ligation mouse model, as described in different vascular injuries such as intimal hyperplasia, arterialized saphenous vein or atherosclerotic plaque (Pullmann et al., 2005).

During the last few years, the involvement of COX-2 downstream prostanoids in vascular remodeling has gained growing attention (Wang et al., 2011b; Zhang et al., 2013; Sparks et al., 2013). Our results demonstrate that PGE_2 and TXA_2 most likely derived from COX-2, are involved in cell migration induced by the combination of AngII+IL-1β. Thus, we found that not only COX-2 but also mPGES-1 and TXAS expressions were increased in VSMC treated with AngII+IL-1β. In addition, although we have no determined PGES and TXAS activities, we observed that cell migration induced by AngII+IL-1β was blocked by pharmacological inhibitors of COX-2 and the TXA_2 and PGE_2 pathways. The effects of both PGE_2 and TXA_2 on VSMC migration were further confirmed by the fact that a PGE_2 analogue and a TP agonist mimicked AngII+IL-1β effects on cell migration, effects that were blocked by EP_1 and EP_3 and TP blockade, respectively. Our results are in agreement with previous findings demonstrating that PGE_2 and TXA_2 induce cell migration in VSMC (through EP_3 receptor) or in endothelial cells (through TP receptor) (Zhang et al., 2013; Bos et al., 2004). The effects of prostanoids on cell migration might be mediated by TN-C, an ECM protein that participates in cell proliferation and migration (Wang et al., 2011b; Yu et al., 2013) and that it is regulated by PGI_2 or PGE_2 (Wang et al., 2011b). Our results demonstrate that not only EP_1 and EP_3 receptors but also TXA_2 participate in the AngII+IL-1β-mediated TN-C induction. An additional relevant finding arising from our study is the fact that the effects of AngII+IL-1β on COX-2, mPGES-1, TXAS and TN-C expression were recapitulated in two animal models of vascular remodeling associated with inflammation and increased cell migration/proliferation (the carotid artery ligation model and the AngII infusion model). Moreover, the vascular remodeling and the increased

TN-C expression induced by AngII were reversed by celecoxib treatment and genetic mPGES-1 deletion, demonstrating that COX-2/mPGES-1/TXAS-derived prostanoids are key mediators of vascular damage/remodeling. Supporting these findings, other authors have demonstrated a role for COX-2, PGE_2 and TXA_2 in animal models of restenosis or hypertension (Wang et al., 2011b; Zhang et al., 2013; Sparks et al., 2013).

Different intracellular signaling pathways are responsible for cell migration. Among the most extensively studied are ERK1/2 and JNK. Both are able to phosphorylate different proteins such as MLCK, focal adhesion kinase or paxilin leading to cytoskeleton reorganization and cell migration (Huang et al., 2004). CaM and pCaMKII are also important mediators of VSMC migration (Scott et al., 2012). CaM/pCaMKII is activated by Gq proteins which are associated to EP_1, EP_3 and TP receptors (Bos et al., 2004; Yamaoka et al., 2009). The participation of EGFR in VSMC migration has been suggested in response to AngII (Mugabe et al., 2010). Interestingly EGFR can be activated directly by TN-C or transactived by EP and TP receptors (Han et al., 2006; Uchiyama et al., 2009). Herein, we demonstrate that ERK1/2, JNK, CaM and pCaMKII and EGFR are key mediators responsible for PGE_2 and TXA_2-dependent VSMC migration.

The results obtained in the first part of this PhD Thesis are summarized in Figure 80. We demonstrate in VSMC a post-transcriptional regulation mediated by ERK1/2-dependent HuR shuttling that underlies the synergistic effect of AngII and IL-1β on COX-2 expression. COX-2-derived TXA_2 and PGE_2 acting on TP and EP_1 and EP_3 respectively, induce TN-C expression and promote cell migration. In addition, increased COX-2, mPGES-1, TXAS, TN-C and HuR expressions were observed in animal models of vascular damage and blockade of COX-2 and mPGES-1 protects the vessel against TN-C expression and altered vessels structure.

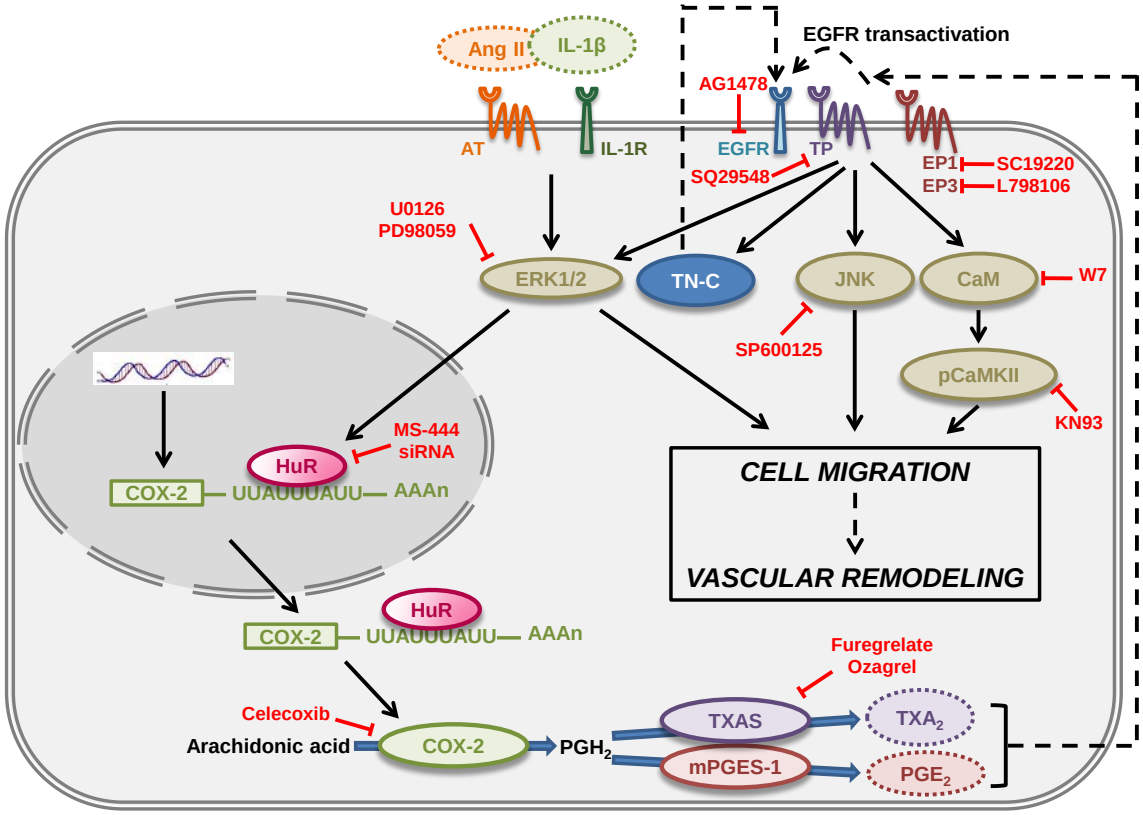

Figure 80. Scheme showing the role of HuR in AngII and IL-1β effects on vascular COX-2 expression and cell migration. *In VSMC AngII and IL-1β synergistically induces COX-2 expression through ERK1/2 pathway. ERK1/2 activation leads to HuR binding to COX-2 3'UTR mRNA and exportation to the cytoplasm where HuR increases COX-2 mRNA stability. COX-2 is translated to protein and converts arachidonic acid in PGH_2 and TXAS and mPGES-1 will transform it in PGE_2 and TXA_2. Both prostanoids through TP and EP1/EP3 increase TN-C expression and would activate EGFR (EGFR transactivation) that will lead to ERK1/2, JNK, CaM/pCaMKII activation ending in cell migration and vascular remodeling. Inhibitors used in the experiments are showed in the scheme with their protein targets marked with bar-headed lines. Dashed lines indicate non-investigated mechanisms.*

In conclusion, these results suggest that HuR and COX-2/mPGES-1/TXAS pathway could be targets to prevent or regress vascular remodeling associated to cardiovascular diseases. However, an intriguing finding that deserves further discussion is the fact that COX-2-selective nonsteroidal anti-inflammatory drugs seem to be associated with an increase of cardiovascular risk through the reduced biosynthesis of endothelial COX-2-dependent PGI_2 (Patrignani and Patrono, 2014). There are a number of reasons that might explain the apparently contradictory effects of blockade of prostanoids pathways between experimental and human studies, but very probably relate to the different animal models, duration or type of the pathology studied or particular inhibitor assayed

in these studies. To overcome the cardiovascular problems of coxibs, in the last few years selective inhibitors of mPGES-1 or specific prostanoids receptors as drug targets with previsible less cardiovascular side effects are being developed (Wang and Fitzgerald, 2010; Korotkova and Jakobsson, 2014). However, the results are far for being conclusive. Interestingly, rediversion of the excess of PGH_2 towards PGI_2 synthase thereby enhancing PGI_2 biosynthesis has been described in mPGES-1 knockout mice (Wang et al., 2006; Wang et al., 2011b), which might provide additional cardiovascular safety compared to COX-2 blockade. Another important consideration is the fact that recent evidences support different and opposing contributions of COX-2 in different cardiovascular compartments (i.e endothelial cells *vs* macrophages) and suggest that targeted blockade of COX-2 to macrophages may conserve their efficacy while limiting cardiovascular risk (Tang et al., 2014). Finally, there is currently some controversy of whether selective and non-selective COX-2 inhibitors provide the same degree of cardiovascular risk (Bhala et al., 2013; Patrignani and Patrono, 2014). In this sense, results of the PRECISION study, an ongoing cardiovascular outcome trial comparing celecoxib, ibuprofen and naproxen, will hopefully shed light on this issue. There is no doubt that results from animals- and cell-based studies similar to the ones presented here are part of a proof of concept and that future clinical trials will definitively address the cardiovascular consequences of selective and non-selective COX-2 blockade in the context of different cardiovascular diseases.

2. HuR IS REQUIRED FOR NOX-1 BUT NOT NOX-4 REGULATION BY INFLAMMATORY STIMULI IN VASCULAR SMOOTH MUSCLE CELLS

Besides COX-2 derived prostanoids, it is well known that NADPH oxidase-derived ROS are implicated in vascular remodeling associated with several cardiovascular pathologies (Xu and Touyz, 2006; Lassègue and Griendling, 2010). The second part of this PhD Thesis provides evidences that in VSMC AngII and IL-1β synergistically induce NOX-1 expression, at least in part, through a HuR-mediated increase in NOX-1 mRNA stability subsequently leading to increased NADPH oxidase activity and ROS production. In contrast,

IL-1β decreases NOX-4 expression and NOX-4-derived H_2O_2 through a transcriptional mechanism involving response elements located in the 192 proximal promoter. Both effects contribute to cell migration triggered by inflammatory mediators and could be implicated in the vascular remodeling associated with cardiovascular diseases.

We observed that IL-1β and AngII induced NOX-1 expression in VSMC, which is in agreement with previous studies performed in the same cell type (Lassègue et al., 2001; Briones et al., 2011; Martín et al., 2012). We also found that the combination of AngII and IL-1β lead to a potentiated NOX-1 induction suggesting that, similarly to COX-2, amplifying mechanisms occur to modulate NOX-1 expression in the presence of several pro-inflammatory stimuli. This effect was dependent, at least in part, on MAPK signaling (ERK1/2, JNK and p38 MAPK) and we also observed that transcriptional and post-transcriptional mechanisms are involved. Specifically, we demonstrate that a post-transcriptional regulatory mechanism mediated by the RNA binding protein HuR participates in the exacerbated NOX-1 induction in VSMC. This is based in the following findings: 1) AngII+IL-1β interfered with the decay of NOX-1 mRNA; 2) AngII+IL-1β increased HuR binding to NOX-1 mRNA; 3) HuR silencing and treatment with the HuR inhibitor MS-444 reduced NOX-1 mRNA stability and AngII+IL-1β-induced NOX-1 mRNA levels. HuR-dependent mRNA stabilization has been suggested to be involved in the post-transcriptional regulation of various pro-inflammatory genes (Meisner and Filipowicz, 2011). However, to our knowledge this is the first report demonstrating that HuR is involved in NOX-1 expression. More importantly, this regulation has a functional role on enzyme activity, since we demonstrate that the synergistically increased NADPH oxidase activity and ROS production induced by AngII+IL-1β, were abolished by NOX-1 and HuR blockade using pharmacological and knockdown approaches. Since NOX-1 has been described to be responsible for $O_2^{\bullet-}$ production and redox signaling in pathological conditions including atherosclerosis, diabetes, and hypertension (Matsuno et al., 2005; Gray et al., 2013), future studies are warranted to determine the potential role of HuR/NOX-1 regulation *in vivo*.

Previous studies suggested that NOX-4 might be a housekeeping gene

due to the presence of GC rich regions in its promoter (Katsuyama et al., 2012). However, modulation of NOX-4 by different stimuli has been described (Chen et al., 2012). TGF-β and lFN-γ induce NOX-4 expression in VSMC (Cucoranu et al., 2005; Manea et al., 2010a), while the effects of AngII and IL-1β on NOX-4 expression are controversial with NOX-4 being up- and down-regulated by these agents in vascular cells (Lassègue et al., 2001; Ellmark et al., 2005; Richard et al., 2009; Briones et al., 2011). We found that IL-1β significantly reduced NOX-4 gene expression with AngII having no additional effect. Our results indicate that IL-1β-mediated NOX-4 decrease is due to a transcriptional repression, involving ERK1/2, JNK and PI3K and the 200-bp region of NOX-4 proximal promoter. Epigenetic mechanisms including DNA methylation or histone deacetylation might explain NOX-4 down-regulation. Our results excluded DNA methylation mechanisms since 5-aza-dC did not modify NOX-4 in VSMC. To our knowledge, very few studies have evaluated the role of HDAC in NOX-4 expression in vascular cells. Sp3 deacetylation has been implicated in basal expression of NOX-4 in endothelial cells (Katsuyama et al., 2011). In VSMC, we observed that HDAC inhibitors decreased NOX-4 mRNA levels and luciferase activity of the NOX-4 proximal promoter in unstimulated cells supporting the role of deacetylation in basal NOX-4 expression. We do not know the exact mechanism but deacetylation of a chromatin region of a repressor or deacetylation of a transcription factor binding to the proximal promoter might be involved. Interestingly, no effect on these inhibitors was observed in IL-1β-treated cells. The fact that basal NOX-4 expression is almost abolished by both trichostatin and SAHA probably prevents further effects of IL-1β. Future investigations are needed to more accurately evaluate the role of HDAC in NOX-4 expression.

We then focused on other mechanisms possibly involved in NOX-4 repression. Thus, we found that cycloheximide, a protein synthesis inhibitor abolished IL-1β-dependent NOX-4 mRNA decrease. In addition, serial deletion analysis of NOX-4 promoter region enclosed IL-1β-dependent down-regulation to the 192 bp proximal region. *In silico* analysis of this promoter region using MatInspector software indicated some candidate repressors (PPAR-γ, Elk-1, Egr-1, Elf-3) that may be contributing to IL-1β-dependent NOX-4 down-

regulation. However, our data exclude the contribution of these transcription factors to the down-regulation of NOX-4 expression induced by IL-1β. Very few studies have examined transcriptional mechanisms involved in NOX-4 down-regulation. Recently, JunD a member of the AP-1 family of transcription factors, has been described as a repressor of NOX-4 expression at vascular level (Paneni et al., 2013); however, the 192 bp proximal region lacks an AP-1 binding site. Further examination is needed to identify and characterize which factor is responsible for this effect. Of interest is the fact that the effects of IL-1β on NOX-4 expression have a functional consequence in ROS production. Thus, IL-1β decreased H_2O_2 production and this effect was reversed by NOX-4 over-expression suggesting that NOX-4 might function as a H_2O_2 generating enzyme as has been previously suggested by other authors (Dikalov et al., 2008; Takac et al., 2013).

Both $O_2^{\bullet-}$ and H_2O_2 are able to induce cell migration (Velarde et al., 2004; Yoo et al., 2011; Al Ghouleh et al., 2013; Schröder, 2014) through actin cytoskeleton reorganization (Stanley et al., 2014). We demonstrate that NOX-1-derived ROS presumably under the influence of HuR, participates in cell migration since the potentiation induced by AngII+IL-1β on VSMC migration was inhibited by pharmacological inhibition and knockdown of both NOX-1 and HuR. Moreover, NOX-4 overexpression further increased IL-1β-induced VSMC migration, suggesting that both NOX-1 and NOX-4 participate in cell migration in inflammatory conditions. Interestingly, as discussed above, we observed that IL-1β decreased NOX-4 expression in VSMC suggesting that NOX-4 might not significantly contribute to IL-1β induced vascular damage. Alternatively, NOX4 down-regulation might favor a dedifferentiated VSMC phenotype which is an important process involved in cell migration (Clempus et al., 2007; Rzucidlo et al., 2007). The role of NOX-1 in vascular remodeling has been previously demonstrated since NOX-1 knockout mice were protected against vascular remodeling induced by wire injury (Lee et al., 2009). However, the role of NOX-4 in vascular damage is controversial. Thus, depending on the pathology or the vascular bed studied, increased, decreased or unchanged NOX-4 expression can be found and some studies suggest NOX-4 as a deleterious protein whereas others demonstrate that NOX-4 is a vascular protective enzyme (Chen

et al., 2012; Touyz and Montezano, 2012; Schröder et al., 2012). Future studies are warranted to further clarify the role of NOX-4 in vascular diseases.

Our study has some limitations and deserves further considerations. We have focused on NOX-1 and NOX-4 because these isoforms appear to be the key vascular NOXs involved in VSMC migration (Lassègue et al., 2012). However, it is well known that activation of other NOXs (NOX-2 and NOX-5) also contribute to $O_2^{\bullet -}$ production in rodent and/or human VSMC (Làssegue et al., 2012). As such, we cannot exclude the possibility that these NOXs may also play a role in the results observed here. In depth studies to further study NOX-2 and NOX-5 regulation, particularly in human VSMC, will elucidate whether these isoforms also play a role in AngII and/or IL-1β-mediated ROS-dependent migration of VSMC. Exact reasons for the differential regulation of NOX-1 and NOX-4 that we observed remain unclear but may relate to localization of different NOXs in distinct intracellular regions (Làssegue et al., 2012). For example, NOX-1 appears to localize primarily in caveolae whereas NOX-4 localizes to the nucleus and focal adhesions (Hilenski et al., 2004). In VSMC, both NOX-1 and NOX-4 participate in cell migration, although via different mechanisms (Làssegue et al., 2012).

The results obtained in the second part of this PhD Thesis are summarized in Figures 81 and 82. We demonstrate that transcriptional and post-transcriptional mechanisms dependent on HuR-mediated mRNA stabilization are responsible for AngII+IL-1β-dependent NOX-1 expression in VSMC (Figure 81). We also demonstrate that IL-1β decreases NOX-4 expression in VSMC through transcriptional mechanisms involving the proximal promoter (Figure 82). Regulation of NOXs is important since NOX-1-derived ROS and NOX-4-derived H_2O_2 participate in cell migration.

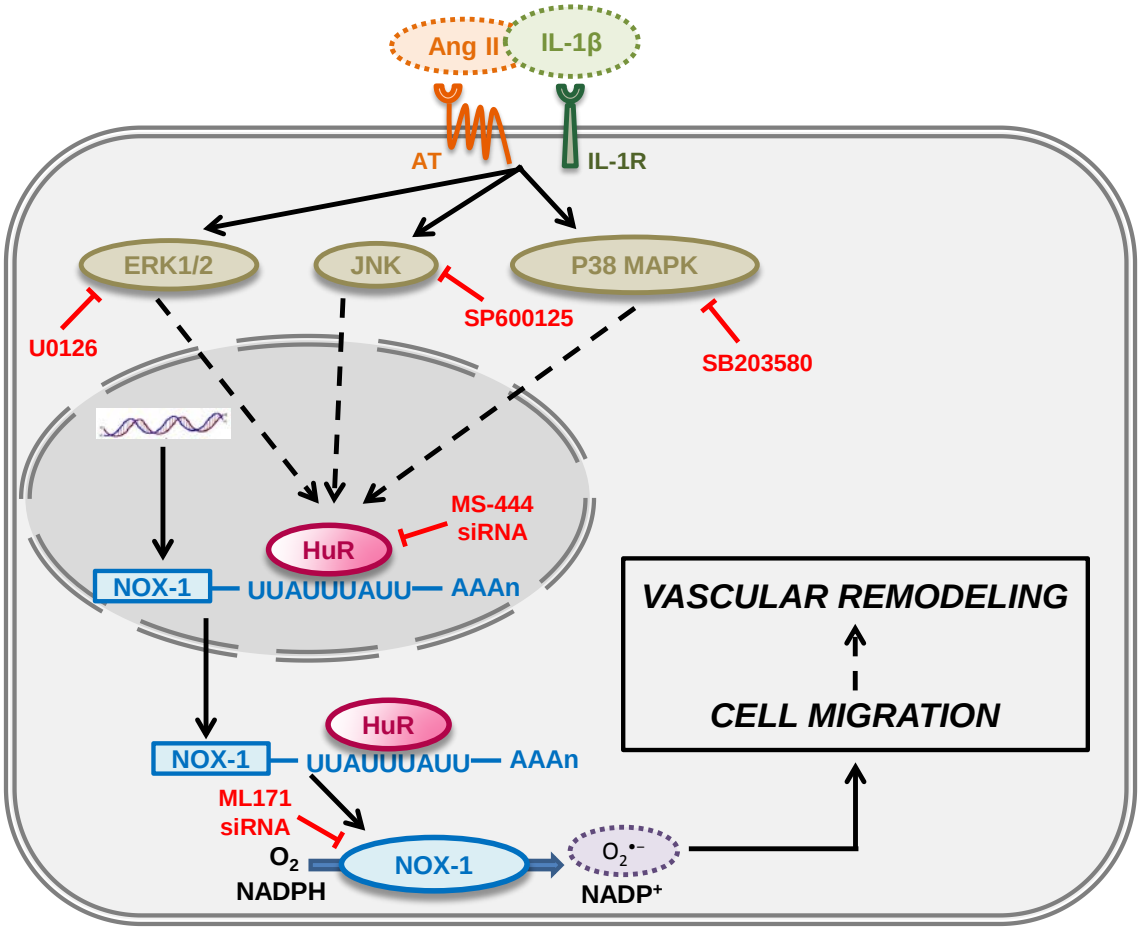

Figure 81. Scheme showing the role of HuR in AngII and IL-1β effects on vascular NOX-1 expression and cell migration. *In VSMC AngII and IL-1β synergistically induces NOX-1 expression through ERK1/2, JNK and p38 MAPK pathways. HuR binds to NOX-1 3'UTR mRNA, increasing NOX-1 mRNA stability. NOX-1 is translated to protein and converts O_2 in $O_2^{\bullet-}$ using NADPH as a cofactor. NOX-1 derived $O_2^{\bullet-}$ is involved in cell migration and possibly in vascular remodeling. Inhibitors used in the experiments are showed in the scheme with their protein targets marked with bar-headed lines. Dashed lines indicate non-investigated mechanisms.*

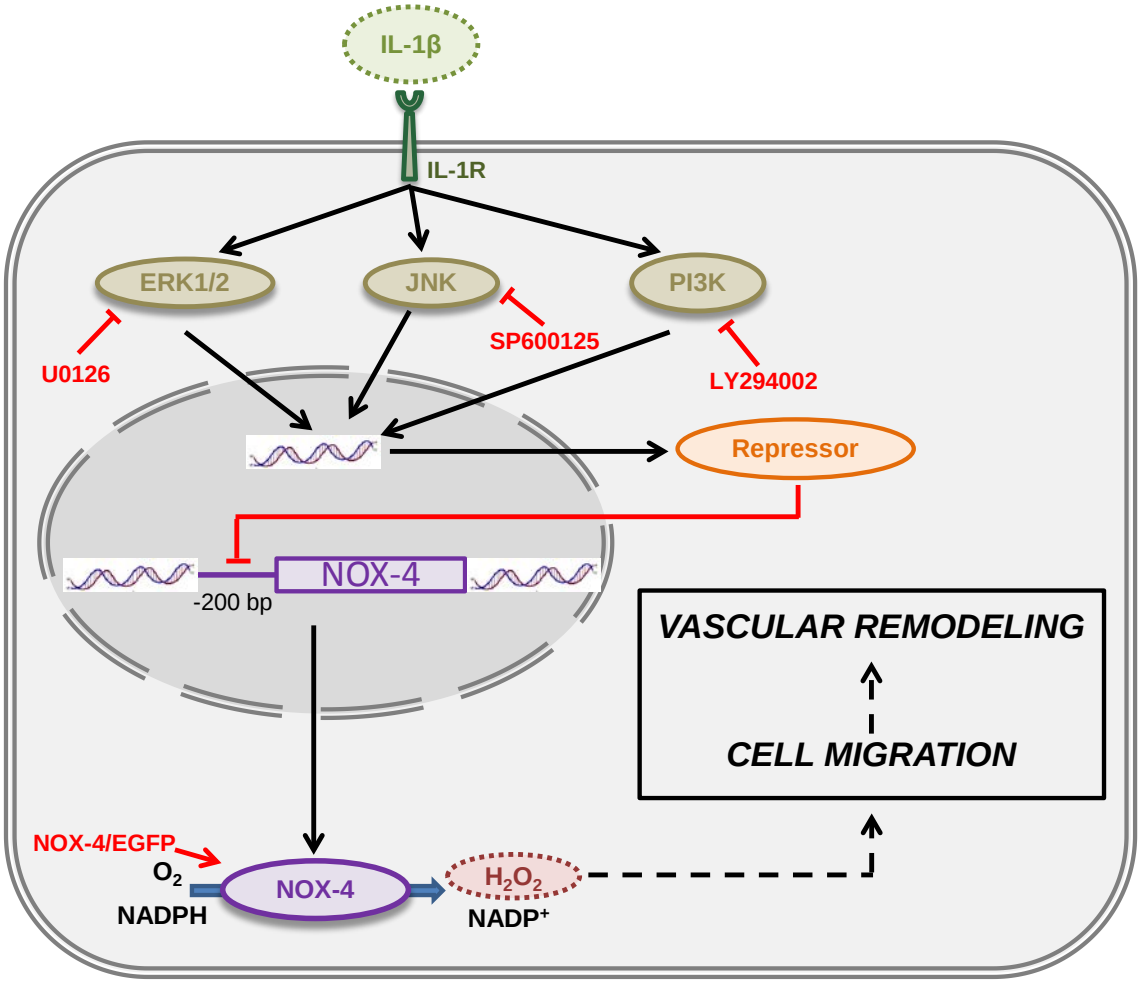

Figure 82. Scheme showing how IL-1β decreases vascular NOX-4 expression and cell migration. *In VSMC IL-1β increases a NOX-4 repressor through ERK1/2, JNK and PI3K pathways. This repressor binds to the proximal promoter (200 bp) and inhibits NOX-4 transcription thus decreasing NOX-4 protein levels. NOX-4 converts O_2 in H_2O_2 using NADPH as a cofactor; this H_2O_2 participate cell migration and possibly to vascular remodeling. Inhibitors used in the experiments are showed in the scheme with their protein targets marked with bar-headed lines and NOX-4/EGFP over-expression is shown with an arrow. Dashed lines indicate non-investigated mechanisms.*

In conclusion, our study further elucidates mechanisms regulating NOX-1 and NOX-4 and indicates how this impacts on functional responses of VSMC. Targeting such mechanisms may provide new approaches to regulating abnormal VSMC migration in vascular injury associated with cardiovascular disease. It is important to highlight that similarly to findings related to the COX-2 pathway, the role of oxidative stress in the clinical context is still unclear. Despite of the extensive literature suggesting the key role of oxidative stress in different aspects of cardiovascular diseases, clinical trials with antioxidants have no provided conclusive and direct evidences between ROS and

cardiovascular diseases. Possible reasons are multiple and relate among other factors with pharmacological, methodological and molecular aspects of ROS biology. Thus, after the clinical failure of most antioxidant trials, NOX inhibitors are the most promising therapeutic option for diseases associated with oxidative stress; unfortunately to date, the selectivity or the pharmacokinetic properties of these compounds is still limited (Altenhöfer et al., 2014). A variety of methods have been developed for ROS detection; however, many of these methods are not exempted of selectivity problems and more research is needed to develop sensitive, specific and reliable biomarkers and assays to assess the redox status of humans in health and disease (Dikalov and Harrison, 2014; Violi and Pignatelli, 2014). Finally, despite of growing information regarding the different NOX isoforms present in human vascular cells, there is still a paucity of information on molecular mechanisms of ROS biology in human tissue (Montezano and Touyz, 2014). Hopefully, future advances in these aspects will provide definitive evidences of the role of oxidative stress in cardiovascular diseases.

3. FINAL CONSIDERATIONS

An increasing body of evidence supports the notion that inflammation plays an important role in the development and progression of cardiovascular diseases. In this context, activation of local RAAS and elevated levels of pro-inflammatory cytokines such as IL-1β play a key role in the structural and functional alterations of the vasculature. HuR is a member of the Hu family of RNA-binding proteins and its activity and function is associated with its subcellular distribution, transcriptional regulation and translational and post-translational modifications. HuR is able to regulate target mRNAs through its interaction with AREs in the untranslated region of target mRNAs. These effects have been extensively investigated in cancer research (Wang et al., 2013) and within this context, this protein is involved in different processes including inflammation, cell proliferation and angiogenesis through the mRNA stabilization of different ILs, TNF-α, cyclins and VEGF (Wang et al., 2013). The role of HuR in cardiovascular diseases is very unknown. Under mechanical and biochemical stresses HuR increases adhesion of monocytes and ICAM-1 and VCAM-1 expression in endothelial cells (Rhee et al., 2010). In this PhD Thesis, we

demonstrate that HuR is involved vascular inflammation and VSMC migration through the regulation of COX-2 and NOX-1 mRNAs, two enzymes involved in inflammation and altered vascular structure. COX-2-derived prostanoids and NOX-1-derived ROS are implicated in vascular remodeling in different cardiovascular pathologies (Wang et al., 2011b; Zhang et al., 2013; Sparks et al., 2013; Lee et al., 2009; Gavazzi et al., 2006; Matsuno et al., 2005). The participation of these enzymes in vascular structure relies on the reported effects of TXA_2, PGE_2 and ROS in promoting cell proliferation and migration (Sachinidis et al., 1995; Nie et al., 2000; Stanic et al., 2010; Wang et al., 2011b; Valente et al., 2012; Al Ghouleh et al., 2013). We provide *in vitro* and *in vivo* data demonstrating that the HuR/COX-2/PGE_2 and TXA_2 pathway is responsible for VSMC migration. Similarly, HuR/NOX-1/ROS also promote cell migration. We have previously demonstrated a reciprocal relationship between COX-2 and NOX which is responsible for hypertension and for hypertension-associated vascular alterations (Martínez-Revelles et al., 2013). Since COX-2 and NOX-1 seems to have shared mechanisms of regulation, the reciprocal regulation of these mediators might have a critical role in several pathologies through a harmful self-perpetuating cascade. However, we did not clearly observe this association in our experimental conditions and a more exhaustive analysis of this reciprocal relationship should be carried out in order to definitively exclude a COX-2/NOX-1 interaction in this context.

Growing evidences suggest that NOX-4 regulation might be different to other NOX and in fact, no a clear line exist between the protective and damaging effects of NOX-4-derived ROS (Lassègue et al., 2012; Konior et al., 2014). NOX-4 has been described as a housekeeping and as an inducible gene (Sturrock et al., 2006; Mittal et al., 2007; Katsuyama et al., 2012). However, it has also been described that NOX-4 expression is reduced by pro-inflammatory stimuli in vascular cells (Ellmark et al., 2005). Our results show that NOX-4 regulation differs radically from NOX-1 regulation; while IL-1β induces NOX-1 expression, NOX-4 expression is reduced. Moreover, IL-1β-induced NOX-4 decrease is mediated by transcriptional repression, demonstrating completely different regulation of both NOXs. Our results also show that NOX-4-derived

H_2O_2 can promote VSMC migration suggesting that alterations in NOX-4 gene regulation might impact on the vasculature.

Conclusions

In this PhD Thesis, we demonstrate that the combination of two inflammatory stimuli AngII and IL-1β which are important for cardiovascular diseases, synergistically induce the expression of two major pro-inflammatory proteins COX-2 and NOX-1. This effect leads to the production of PGE_2, TXA_2 and ROS that have a key role in VSMC migration and hence in vascular remodeling in cardiovascular diseases. We also demonstrate that the RNA binding protein, HuR, is key to increase the mRNA stability of both COX-2 and NOX-1 thus participating in cell migration induced by inflammatory stimuli. Finally, we detected differential regulation of NOX-1 and NOX-4 being the latter decreased by IL-1β through transcriptional mechanisms involving the proximal promoter. The partial conclusions are as follows:

1. In VSMC AngII and IL-1β synergistically induce COX-2 and TN-C expression and cell migration.

2. ERK1/2-mediated HuR activation is involved in the synergistic effect of AngII and IL-1β on COX-2 expression and cell migration.

3. PGE_2 and TXA_2 derived from COX-2 participate in cell migration induced by AngII and IL-1β.

4. Animal models of vascular remodeling display increased HuR, COX-2, mPGES-1, TXAS and TN-C expression. COX-2 and mPGES-1 blockade reverse vascular hypertrophy and TN-C expression.

5. AngII and IL-1β synergistically induce NOX-1 expression and activity through an increase of NOX-1 mRNA stability mediated by HuR.

6. NOX-1-derived-ROS participate in AngII+IL-1β induced cell migration.

7. IL-1β-induced NOX-4 down-regulation is mediated by an inducible repressor which binds to the 200 bp proximal promoter.

8. All these effects could contribute to the vascular damage induce by AngII and IL-1β in the vascular wall.

Our study further elucidates mechanisms regulating COX-2 and NOXs and indicates how this impacts on functional responses of VSMC. Specifically, HuR-dependent regulation of COX-2 and NOX-1 in VSMC is a novel mechanism that might contribute to vascular remodeling in different cardiovascular diseases. We believe that targeting the HuR/COX-2/NOX-1 pathways may provide new approaches to regulating abnormal VSMC migration in vascular injury associated with cardiovascular disease.

Conclusiones

En esta Tesis Doctoral demostramos que la combinación de dos estímulos inflamatorios, AngII e IL-1β, los cuales son importantes en enfermedades cardiovasculares, induce la expresión sinérgica de dos de las principales proteínas pro-inflamatorias, COX-2 y NOX-1. Este efecto da lugar a la producción de PGE_2, TXA_2 y especies reactivas de oxígeno, las cuales tienen un papel fundamental en la migración de las CMLV y, por tanto, en el remodelado vascular en enfermedades cardiovasculares. También demostramos que la proteína de unión a RNA, HuR, es clave en el incremento de la estabilidad de los mRNAs de COX-2 y NOX-1, participando así en la migración celular inducida por estímulos inflamatorios. Finalmente, demostramos una regulación diferente de NOX-1 y NOX-4 siendo ésta última disminuida por IL-1β a través de mecanismos transcripcionales asociados al promotor proximal. Las conclusiones parciales son las siguientes:

1. En CMLV, AngII e IL-1β inducen sinérgicamente la expresión de COX-2 y TN-C así como migración celular.

2. La activación de HuR mediada por ERK1/2 está implicada en el efecto sinérgico de AngII e IL-1β sobre la expresión de COX-2 y la migración celular.

3. PGE_2 y TXA_2 derivados de COX-2 participan en la migración celular inducida por AngII e IL-1β.

4. Modelos animales de remodelado vascular muestran un incremento en la expresión de HuR, COX-2, mPGES-1, TXAS y TN-C. El bloqueo de COX-2 y de mPGES-1 revierte la hipertrofia vascular y la expresión de TN-C.

5. AngII e IL-1β inducen sinérgicamente la expresión y actividad de NOX-1 a través de un incremento en la estabilidad del mRNA de NOX-1 mediado por HuR.

6. Las especies reactivas de oxígeno derivadas de NOX-1 participan en la migración celular inducida por AngII e IL-1β.

7. La disminución de NOX-4 inducida por IL-1β está mediada por un represor inducible que se une en las 200 bp del promotor proximal.

8. Todos estos efectos podrían contribuir al daño vascular inducido por AngII e IL-1β en la pared vascular.

Nuestros estudios elucidan algunos de los mecanismos reguladores de COX-2 y NOXs y muestran el impacto que éstos tienen sobre las respuestas funcionales de las CMLV. Específicamente, la regulación de COX-2 y NOX-1 dependiente de HuR en CMLV es un nuevo mecanismo que contribuiría al remodelado vascular en diferentes enfermedades cardiovasculares. Creemos que las rutas HuR/COX-2/NOX-1 podrían ser dianas terapéuticas para el desarrollo de nuevas aproximaciones que controlen la migración anormal de las CMLV en daño vascular asociado con enfermedades cardiovasculares.

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